Vol. XLIV No. 1

January 1955

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

CONTENTS OF THIS ISSUE

Synthetic Riboflavin-5'-Phosphate 1
E. De Ritter, J. Scheiner, F. W. Jahns, L. Drekter, and *S. H. Rubin*

The Relative Efficacy of Various Ointment Bases as Antiseptic Vehicles 5
Charles K. Kolstad and *C. O. Lee*

An Evaluation of the Stability of Buffered Erythromycin Ointments 7
Harold Sheinaus and *C. O. Lee*

Beta-Phenylethyl Alcohol as a Preservative for Ophthalmic Solutions 9
I. W. Grote and *Maribelle Woods*

A Collaborative Study on the Use of Mice in Acute Toxicity Testing 11
O. F. Swoap

A Note on Tablet Disintegration with Starch 16
Lemuel C. Curlin

A Synthesis of Pseudopelletierine 17
Blake F. Putney and *Taito O. Soine*

DDT vs. Wildlife—Relationships Between Quantities Ingested, Toxic Effects and Tissue Storage 22
James B. DeWitt, James V. Derby, Jr., and *George F. Mangan, Jr.*

Buffered Ophthalmic Solutions of Silver Nitrate 25
Walter F. Charnicki and *Mary L. Kober*

The Chronic Oral Toxicology of the Polyethylene Glycols 27
Henry F. Smyth, Jr., Charles P. Carpenter, and *Carrol S. Weil*

A Note on Polarography and Chromatography of Some Commercially Available Chlorophyllins 30
Walter L. Wuggatzer and *John E. Christian*

Preparation and Properties of 8-Chlorotheophylline Salts of Some Alkaloids 32
M. L. Robinette, Jr. and *F. W. Bope*

Studies in the Stabilization of Folic Acid in Liquid Pharmaceutical Preparations 34
Robert P. Tansey and *George H. Schneller*

The Saponins of Pachyrrhizus Erosus (L) Urban 38
Ralph F. Shangraw and *E. V. Lynn*

A Pharmacognostical Study of Pyrularia Pubera Michx 39
Edward P. Claus

A Study of Thermoelectric Methods for Determining Isotonicity 43
Frank M. Goyen and *Donald Reck*

Derivatives of Alginic Acid 48
J. E. Wintter, W. M. Lauer, and *P. A. Foote*

The Effect of Germination on the Alkaloid Content of Nigella damascena Seed 52
V. E. Tyler, Jr.

Microtoxicology XI: Identification and Differentiation of Seven New Synthetic Antispasmodics 54
Thomas J. Haley and *George L. Keenan*

Central Depressant Properties of Uracil and Related Oxypyrimidines 56
Duane G. Wenzel and *Moreno L. Keplinger*

Anti-Metrazol® Efficacy of Combinations of Meparfynol and Anticonvulsant Drugs 59
David A. Brodie, Arthur Tye, and *John W. Nelson*

A Note on the Use of Amberlite® IRC-50 for the Purification of Nonaqueous Solvents 61
Leo Levi, Leslie G. Chatten, and *M. Perlmanowski*

Book Notices 62

Advertising ..... Cover pages 2, 3, 4, and pages I, II, III, VI, VII, and VIII

New and Nonofficial Remedies ..... IV & V

Copyright 1955, by the American Pharmaceutical Association

USE
SUPERIOR
INGREDIENTS
for
SUPERIOR
PRODUCTS

write ON YOUR BUSINESS LETTERHEAD TO . . .

Amerchols® are natural, non-ionic OINTMENT BASES AND EMULSIFIERS which contain *Lanolin Cholesterols in their Most Active Form.*

These stable cholesterol and multisterol surfactants induce rapid drug release, promote optimum healing rates, and are safe for the most delicate tissues. We know of no case of an allergy due to an AMERCHOL.*

Modulan™ is a chemically modified LANOLIN EMOLLIENT with unique new properties for use in OINTMENTS AND EMULSIONS. It imparts waxy, protective hydrophobic films; is oil soluble and compatible with O/W emulsions, soaps and shampoos. Clinical investigations indicate that MODULAN IS HYPO-ALLERGENIC.*

*References, technical data and suggested formulations are available from our research laboratories.

AMERICAN CHOLESTEROL PRODUCTS
• INCORPORATED •
MILLTOWN • NEW JERSEY

AMERICAN CHOLESTEROL PRODUCTS, INC. AMERCHOL

Scale illustrated is our No. 540 Popular Model
Capacity—4 oz. (120 grams)
Sensibility Reciprocal—1/10 grain
Finish—Genuine light mahogany with chrome plated parts
Price—$145.00

PROMPT DELIVERY
on this as well as other class A scales
No. W35.....$110.00
No. W33..... 98.00
All With Stainless Steel Plans

Our scales have no bands or wires to stretch or break

Still the best and lowest priced scale made

HENRY TROEMNER
Manufacturer of the Finest Scales for the Druggist Since 1840
911 Arch Street
Philadelphia 7, Pa.
(*Send for Circular A-A*)

MASSACHUSETTS COLLEGE OF PHARMACY ONE YEAR ONE TWO

The Massachusetts College of Pharmacy
Longwood Avenue, Boston

Graduate study in Boston has always appealed strongly to students in the fields of medicine and public health. The environment is stimulating and attracts students from all parts of the world. Library, laboratory, hospital, and other facilities for graduate work are outstanding in this area.

The Massachusetts College of Pharmacy is located in this great medical and educational center. It offers unusual opportunities to graduates of colleges of pharmacy who are interested in further study in pharmacy, pharmaceutical chemistry, and pharmacognosy.

For additional information, write to HOWARD C. NEWTON, Dean

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

SCIENTIFIC EDITION

Committee on Publications

Don E. Francke, *Chairman*
Walter M. Chase
Robert P. Fischelis
F. Royce Franzoni
Hugo H. Schaefer

Editorial Advisory Board

John E. Christian
Walter C. Gakenheimer
George P. Hager
Ewart A. Swinyard
Elmer B. Vliet
H. W. Youngken, Jr.

JUSTIN L. POWERS, EDITOR
SAMUEL W. GOLDSTEIN, ASSISTANT EDITOR
VIRGINIA F. WILLS, EDITORIAL ASSISTANT

NOTICE

PAPERS FOR SECTION MEETINGS OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

The next annual meeting of the American Pharmaceutical Association will be held in Miami Beach, Florida, May 1 to 7, 1955. Members planning to present papers before any section during this meeting are earnestly requested and strongly urged to notify the appropriate Section secretary of their intention promptly. Titles and abstracts (not to exceed 200 words) of papers should be submitted by March 1, 1955, or preferably earlier, in order to insure a place on any section program. Send titles and abstracts of papers to:

SECTION SECRETARIES

SCIENTIFIC
ARTHUR J. MCBAY,
Massachusetts College of Pharmacy
179 Longwood Avenue
Boston 15, Mass.

PRACTICAL PHARMACY
SAMUEL W. GOLDSTEIN
2215 Constitution Avenue, N. W.
Washington 7, D. C.

HISTORICAL PHARMACY
GEORGE B. GRIFFENHAGEN
Smithsonian Institution
Washington 25, D. C.

EDUCATION AND LEGISLATION
HUGH FERGUSON
University of New Mexico
College of Pharmacy
Albuquerque, N. Mex.

PHARMACEUTICAL ECONOMICS
NOEL M. FERGUSON
3337 Prospect
Houston 4, Texas

The Journal of the American Pharmaceutical Association is published monthly by the American Pharmaceutical Association in two editions: Scientific Edition and Practical Pharmacy Edition, under the supervision of the Committee on Publications of the Council of the Association. Publication office: 20th and Northampton Streets, Easton, Pennsylvania.

Editorial and Executive Offices—2215 Constitution Avenue, N.W., Washington 7, D. C. Robert P. Fischelis, Secretary and General Manager.

Annual Subscription—Journal of the American Pharmaceutical Association, Practical Pharmacy Edition and Scientific Edition combined: United States, Canada, Pan America, Spain, and Philippines, $10.00; other foreign, $12.00.

Journal of the American Pharmaceutical Association, Scientific Edition alone: United States, Canada, Pan America, Spain, and Philippines, $6.00, other foreign, $7.00.

Journal of the American Pharmaceutical Association, Practical Pharmacy Edition alone: United States, Canada, Pan America, Spain, and Philippines, $5.00, other foreign, $6.00.

Members of the American Pharmaceutical Association receive the combined subscription as a part of their annual dues. Members may request omission of either edition, but this will not reduce the amount of the annual dues.

Single Copies—Journal of the American Pharmaceutical Association, Practical Pharmacy Edition: United States, Canada, Pan America Spain, and Philippines, $0.50; other foreign, $0.60. Scientific Edition: United States, Canada, Pan America, Spain, and Philippines, $0.60; other foreign, $0.70.

Back issues of unbound Journals same price as current volume.

Subscription rates are subject to change without notice.

Change of Address—Members and subscribers should bear in mind that second class matter is not forwarded by the Post Office and should supply both the Post Office and the American Pharmaceutical Association with any change of address immediately upon its occurrence.

Claims for missing numbers will not be allowed if received more than 60 days after the date of issue, or if loss was due to failure to give 30 days' notice of change of address. Claims asserting that Journals are "missing from files" cannot be allowed. The Association cannot accept responsibility for foreign delivery when its records indicate that shipment has been made.

Entered as second class matter January 23, 1917, at the Post Office at Easton, Pennsylvania, under the Act of March 3, 1879, as 24 times a year; Scientific Edition monthly on the 5th. Practical Pharmacy Edition monthly on the 20th. Acceptance for mailing at a special rate of postage provided for in Section 1103, Act of October 3, 1917, authorized July 10, 1918.

NOTICE TO AUTHORS OF PAPERS

Manuscripts.—All manuscripts submitted to the Editor or presented to the Sections of the ASSOCIATION should be ready for publication, typewritten with double spacing on one side of good quality paper 8.5″ x 11″ in size and with 1″ to 1.5″ margins. In addition to the original typewritten copy, one or more carbon copies should be submitted in order to facilitate examination by members of the Editorial Advisory Board or other referees. Each manuscript should be accompanied by an abstract of not more than 200 words which includes a summarization of conclusions and recommendations. All pages in manuscripts, including tables but not figures, should be numbered consecutively. Previously published and readily accessible procedures which have been followed in experimental work should not be made a part of the manuscript, but should only be referred to by appropriate literature references.

Form of Presentation.—Titles of manuscripts should be kept as short as possible and preferably restricted to one line. The name of the laboratory in which the work described was done should be placed in a footnote at the end of the first page. Each manuscript should begin with one or more introductory paragraphs indicating the intention and meaning of the investigation and the method of procedure. The introduction should be followed by a report of the essential details of the experimental work, and a discussion or interpretation where necessary. Finally there should be a brief summary of conclusions and recommendations. Authors may find it helpful to arrange manuscripts to conform to the style of presentation as illustrated in papers on analogous subjects published in recent issues of THIS JOURNAL.

In preparing manuscripts for publication in THIS JOURNAL, center headings should be avoided as far as possible. Side headings followed by a period and dash should be indented and run into the part of the text to which they apply. Such side headings may be used as freely as desired. Literature citations should be numbered consecutively in order of appearance in the manuscript and should be indicated by full-sized Arabic numerals enclosed in parentheses. If a literature reference is cited more than once in a manuscript, subsequent citations should be indicated by the first number assigned. References to footnotes throughout the text should be numbered consecutively by superscript Arabic numerals, but such references in each table should be designated by superscript lower case letters beginning with "a" and continuing in natural order. Literature citations should be grouped at the end of the manuscript under the heading "References." All bibliographic references should be checked with the original publications. The citations should be numbered consecutively with full-sized Arabic numerals enclosed in parentheses corresponding to the numbers used in the text. The names of all periodicals cited in the list of references must be abbreviated in accordance with abbreviations given by **Chemical Abstracts** in its "List of Periodicals Abstracted." The sequence followed in the citations must be as follows:

(1) Doe, J. B., *Am. J. Physiol.*, **79**, 289(1927).

References to books should be in the order given, (1) author's surname and initials, (2) title of the book, (3) volume, (4) edition, (5) name of publisher, (6) place of publication, (7) year of publication, and (8) page number. A complete book reference may be illustrated as follows:

(1) Gilman, H., "Organic Chemistry," Vol. 2, 2nd ed., John Wiley and Sons, New York, 1943, pp. 1236, 1252.

Arabic numerals must be used to designate all definite weights, measurements, percentages, and degrees of temperature unless occurring at the beginning of a sentence, in which case the numeral and measurement are spelled out. Periods of time should be written in words. Unusual characters and Greek letters should be written plainly and legibly or should be explained by notes on the margin of the page. Fractions should be written with a slant line. Standard abbreviations should be used whenever weights and measures are stated in the metric system, e. g., 10 Gm., 2.5 cc., etc. The forms to be used are: cc., Kg., Gm., mg., mm., cm., and L.

Figures.—All drawings should be made in India ink, preferably on white tracing paper or cloth. Original drawings are preferable, but clear, well-prepared glossy photographic prints are acceptable. The size of illustrations should not exceed the 8.5″ x 11″ manuscript size. They should be designed for reduction to the width of one column (2.75″) or full page width (5.5″). Thus, height rather than width should be accentuated where possible.

Numbers and measurements, etc., which are to appear on the axes of the drawing should be indicated in pencil outside the axes, in order that they may be set in type. Captions and legends should be placed *below* the diagram (not on it), or, if lengthy, collected on a separate typewritten list, identifying each by its proper figure number and including whatever acknowledgments are necessary.

Photographs should never be rolled, and paper clips should not be used unless guarded by a piece of paper folded several times to prevent an indentation in the photograph. All drawings and photographs should be protected from bending and from the chance of having their edges torn while in transit. Do not write on the back of a photograph. Any indentation in the photograph, however slight, will be likely to show as a blemish in the reproduction. All figures submitted must be referred to in the text of the manuscript, and should be numbered consecutively with Arabic numerals, e. g., Fig. 1, Fig. 2, etc. Kymograph tracings should not be submitted with the original experimental legends which are likely to have been hastily or carelessly inscribed. Authors may find it worth while to employ a professional draftsman to make the drawings in proper form for reproduction.

Tables.—Tables should be carefully constructed so that the data presented may be understood easily. The indiscriminate use of tables should be avoided. Tables should be constructed so as to occupy fully a single column (2.75″) which will take about 40–50 characters and spaces. Tables which require two columns are also acceptable. Interpretative statements should not be written on the body of a table but should be explained in footnotes. Column heads should be made as small as possible and standard abbreviations should be used liberally. Data which may easily be deduced by simple arithmetic from data in another column should not be included. All tables should be referred to in the text of the manuscript. Every table and every column should be supplied with an appropriate heading. It is not necessary to place tables on separate sheets, and in fact it is preferable to intersperse them in the manuscript. Tables should be numbered consecutively using Roman numerals. The table number and the title should be placed in a continuous heading above the data presented. The preparation of a clear and concise table of data requires much planning and infinite care.

DISPLAY the A.PH.A.

Code of Ethics

A Mark of Professional Prestige

Now available. Beautifully Printed in 3 Colors

ON LAMINATED BOARD

12" x 18", washable, permanent, "easled" for counter display, "ringed" for wall hanging. Postpaid **$2.50**

ON HEAVY STOCK

suitable for framing, 10" x 16". Postpaid **$1.00**

American Pharmaceutical Association

2215 Constitution Ave., Washington 7, D.C.

I enclose check (or money order) for $.....
for which please send me.....copies of the A.PH.A. Code of Ethics. ( ) Laminated Board ( ) Heavy Stock

Name.....

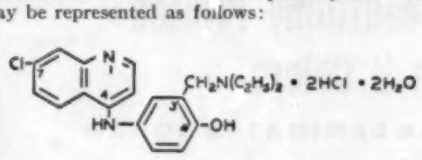
Address.....

.....

NNR

PRODUCTS RECENTLY ACCEPTED BY THE A. M. A. COUNCIL ON PHARMACY AND CHEMISTRY

AMODIAQUIN HYDROCHLORIDE.—Camoquin Hydrochloride (Parke, Davis).—$C_{20}H_{22}ClN_3O \cdot 2H_2O$. —M. W. 464.81—4-(7-Chloro-4-quinolylamino)-α-diethylamino-*o*-cresol dihydrochloride dihydrate.—7-Chloro-4-(3′-diethylaminomethyl-4′-hydroxyanilino)quinoline dihydrochloride dihydrate.—The structural formula of amodiaquin hydrochloride may be represented as follows:



Actions and Uses.—Amodiaquin hydrochloride, a synthetic antimalarial agent, is equal to chloroquine phosphate in antimalarial potency and has the same relatively low toxicity. Like chloroquine, the activity of amodiaquin is limited to the erythrocytic stages of malaria (parasitemia). The drug is capable of producing a radical cure only for infection caused by Plasmodium falciparum; it abolishes the acute attack and eradicates the infection. In infections caused solely or complicated by P. vivax or P. malariae, it is ineffective as a prophylactic against primary infection or relapses but is effective for the treatment of acute attacks to relieve clinical symptoms, to control parasitemia, and to increase the interval between treatment and relapse. Amodiaquin hydrochloride therefore is indicated as a suppressive agent in areas of endemic malaria.

Amodiaquin hydrochloride is rapidly and almost completely absorbed from the gastrointestinal tract. It is distributed unequally in body tissues, where it is present in higher concentrations than in plasma; its concentration in erythrocytes is about twice that found in plasma. It is also concentrated in the spleen, liver, and kidneys. The drug is retained in the tissues from which it is gradually liberated into the blood stream; it is slowly excreted in the urine after administration is discontinued. Clinical side-effects are chiefly those of the gastrointestinal tract (nausea, vomiting, salivation, and diarrhea) and of the central nervous system (incoordination, spasticity, and convulsions), but these seldom are encountered at therapeutic dosage levels. It does not produce discoloration of the skin. Complications have not been encountered in the presence of kidney or liver disease, nor during pregnancy.

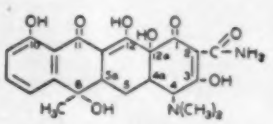
Dosage.—Amodiaquin hydrochloride is administered orally. Dosage is expressed in terms of the base. For the treatment of acute attacks of malaria, the usual single dose for adults is 0.4 to 0.6 Gm. The usual dosage for children, given either as a single dose or in three divided doses at twelve hour intervals, is as follows: one to two years, 0.1 Gm.; three to five years, 0.2 Gm.; six to fifteen years, 0.4 Gm.; fifteen years and above, adult dose. For suppression of endemic malaria, the usual dosage for adults is 0.4 to 0.6 Gm. administered once every two weeks; for children, the dosage should be reduced according to age and spaced at the same interval.

Tests and Standards.—

Physical Properties: Amodiaquin hydrochloride is a yellow, odorless, bitter, crystalline solid, m.p. 150–160° (with decomposition). It is soluble in water; sparingly soluble in alcohol; and very slightly soluble in benzene, chloroform, and ether. The pH of a 1% solution is 4.0–4.8.

[For more information regarding this monograph see *J. Am. Med. Assoc.*, **156**, 1328(December 4, 1954).]

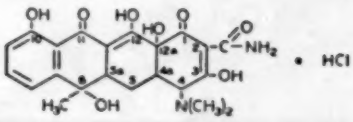
TETRACYCLINE.—Tetracyn (Pfizer).—4-Dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide. Tetracycline is an antibiotic isolated from the elaboration products of certain Streptomyces species when the organism is grown on suitable culture media. It is also prepared by the catalytic halogenation of chlortetracycline (Aureomycin) or oxytetracycline (Terramycin). It differs from the former only in the replacement of the chlorine atom in the structure by a hydrogen atom, and from the latter only in the replacement of one hydroxyl group by a hydrogen atom. The structural formula of tetracycline may be represented as follows:



Actions and Uses.—Tetracycline, the base, has the same actions and uses as tetracycline hydrochloride. (See the monograph on tetracycline hydrochloride.)

Dosage.—Tetracycline is administered orally. The dosage is the same as for the hydrochloride, which also is expressed in terms of the base.

TETRACYCLINE HYDROCHLORIDE.—Achromycin Hydrochloride (Lederle).—Tetracyn Hydrochloride (Pfizer).—4-Dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide hydrochloride. Tetracycline is an antibiotic isolated from the elaboration products of an unidentified species of Streptomyces when the organism is grown on suitable culture media, or is obtained by catalytic reduction of chlortetracycline (Aureomycin) or oxytetracycline (Terramycin). It differs from the former only in the replacement of the chlorine atom in the structure by a hydrogen atom, and from the latter only in the replacement of one hydroxyl group by a hydrogen atom. The structural formula of tetracycline hydrochloride may be represented as follows:



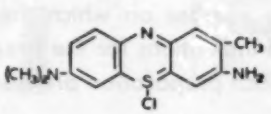
Actions and Uses.—Tetracycline hydrochloride has actions and uses similar to those of chlortetracycline and oxytetracycline. Clinical evidence indicates that it is useful in the treatment of infections produced by bacteria that have been noted to be susceptible to the antibacterial effects of either of those antibiotics. Information relative to antiviral action against viruses is currently lacking as is also knowledge concerning its effect upon rickettsia. No antifungal activity has been demonstrated for tetracycline. An important finding is that if micro-organisms are resistant to chlortetracycline or oxytetracycline, they tend to be resistant to tetracycline also; however, clinical studies have shown that tetracycline may be effective after treatment with chlortetracycline or oxytetracycline has proved ineffective.

Tetracycline has the same ranges of experimental toxicity, both acute and chronic, that have previously been described for chlortetracycline and oxytetracycline. It is more stable in solution and has a solubility similar to that of oxytetracycline; its solubility is greater than that of chlortetracycline. Following single or repeated doses of tetracycline, its concentration in the blood appears to be at a higher level and is better sustained than has been noted for its predecessors. Tetracycline also passes through the essentially normal blood-brain barrier more easily, so that higher concentrations of this compound have been found in the spinal fluid than of either of the other two agents. Tetracycline is excreted in the urine and in the stool (following oral ingestion) in a similar manner and retains its antibacterial activity in the urine and in the gastrointestinal contents. It is not inactivated by blood serum.

Tetracycline produces nausea, vomiting, and loose stools, but the evidence available indicates that these reactions occur less frequently than with the use of either chlortetracycline, or oxytetracycline. Likewise, tetracycline may allow superinfection of the colon with bacteria and yeasts that are resistant to its antibiotic effects.

Dosage.—Current information indicates that the dosages of tetracycline hydrochloride for varying degrees of illness should be the same as those employed when either chlortetracycline or oxytetracycline is used.

TOLONIUM CHLORIDE.—Blutene Chloride (Abbott).—$C_{15}H_{15}ClN_2S$.—M. W. 305.83.—3-Amino-7-dimethylamino-2-methylphenazathionium chloride. —The structural formula of tolonium chloride may be represented as follows:



Actions and Uses.—Tolonium chloride, known as a dye by the name toluidine blue O, exhibits *in vitro* antiheparin activity. In animals the coagulation times of blood samples known to contain an excess of heparin can be returned to normal by the addition of small amounts of the dye. Clinically, the systemic administration of the dye reduces the bleeding tendency in certain hemorrhagic conditions associated with excessive amounts of heparinoid substances in the blood. Excess of these substances can be determined by a simplified method of protamine titration using protamine sulfate, a known antiheparin compound. A protamine sulfate titration value of 0.14 mg. is considered the upper limit of normal.

Tolonium chloride is useful in the treatment of idiopathic functional uterine bleeding: menorrhagia or hypermenorrhea (abnormally profuse or prolonged menstruation) and menometorrhagia (excessive or prolonged menstruation and intermenstrual bleeding). Approximately 80% of patients with idiopathic uterine bleeding have elevated protamine titration values, and 75 to 80% of the patients in this category respond to the dye. In patients treated empirically (not selected on the basis of elevated protamine values), the dye reduces bleeding in about 65%. The mechanism of action has not been explained in uterine bleeding that is not associated with elevated protamine titration but that nevertheless responds to the dye. The dye should not be used for the treatment of abnormal uterine bleeding until adequate examination and study have ruled out malignant lesion as the cause and, when so used without protamine titration, only if all other organic diseases have been ruled out.

Tolonium chloride has been demonstrated to have a low order of toxicity in experimental animals; no changes in coagulation time or capillary fragility have been observed. Extremely high doses injected into dogs produce hemolysis, leukocytosis, and thrombosis, but these effects have not been encountered with therapeutic doses in man. Staining of internal organs may be apparent for a period of time following systemic administration of the dye, but no tissue damage has been attributed to this effect. The urine of patients receiving treatment becomes pale blue-green. Therapy also may be associated with such side-effects as nausea, burning on urination, and tenesmus, but these are usually of minor importance in patients consuming adequate fluids. Since the side-effects may respond to increased fluid intake, decreased dosage, or both, it is rarely necessary to discontinue therapy.

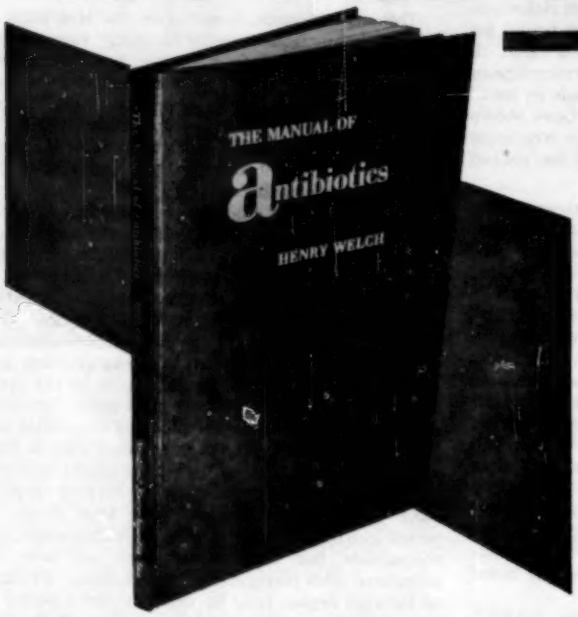
Dosage.—Tolonium chloride is administered orally. The usual dosage is 0.2 to 0.3 Gm. daily. For the treatment of menorrhagia, the drug is administered with meals during the menstrual period; for the prevention of menorrhagia, it is administered for five or six days prior to the estimated time of the menses. In menometrorrhagia, medication may be extended over two or three menstrual periods.

Tests and Standards.—

Physical Properties: Tolonium chloride is a green, crystalline powder with a bronze luster. It is slightly soluble in alcohol, very slightly soluble in chloroform, and practically insoluble in ether. The approximate amount that dissolves at 25° in water to form 100 ml. of solution is 4 Gm.

[For more information regarding this monograph see *J. Am. Med. Assoc.*, 156, 1329(December 4, 1954).]

1954-55 MANUAL



For the first time, there are gathered between the covers of one book a list of the preparations, therapeutic index, generic and trade names, and names and addresses of producers of all existing antibiotics and their preparations.

This information, prepared under the editorial direction of Henry Welch, Ph.D., and published by the Medical Encyclopedia, Inc., is distributed exclusively by the American Pharmaceutical Association.

Lists Ingredients, Trade Names, Indications, and Producers

Practicing pharmacists will welcome this authoritative list of antibiotics and their products, complete as of the date of publication. It provides the list of antibiotics in alphabetical order with the active ingredients, the trade names, the indications and the names of the producers.

For ready reference there is an index of trade names in this field, an index of generic terms applied to antibiotics, and an index of the manufacturers of these products, giving the name of the firm and the address.

To identify a trade name it is only necessary to check the trade name index. Opposite the trade name in question is the page number on which the generic term is given, along with the active ingredients and indications for the preparation. All other trade names that have been assigned to each preparation are also shown.

OF ANTIBIOTICS . .

Multiplicity of Trade Names

In the preface to this manual, Dr. Henry Welch, the author, states: "Over the past few years the number of trade names used for antibiotic preparations has increased tremendously. For example, one single penicillin preparation has as many as thirty-six trade names. In fact, the number of trade names for the same drug is limited only by the number of manufacturers. Because of this multiplicity of trade names it has become practically impossible for the physician, pharmacist, and others engaged in the use of these drugs to remember from the trade name just what the composition of the product is."

The American Pharmaceutical Association is pleased to make this manual available for general distribution as a service to the pharmaceutical and medical professions and to the drug industry.

$2.50—Less 20% Discount to A.Ph.A. Members

The price of the manual is $2.50. A discount of 20 per cent is allowed members of the American Pharmaceutical Association in good standing.

All orders for this publication should be addressed directly to the American Pharmaceutical Association, 2215 Constitution Avenue, N.W., Washington 7, D. C., **and should be accompanied by check or money order to cover the cost, which includes mailing.**

MAIL COUPON Now!

American Pharmaceutical Association S
2215 Constitution Ave., N. W., Washington 7, D. C.

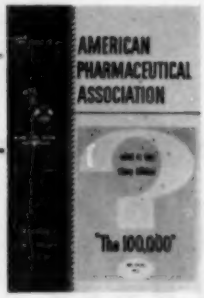
Gentlemen:

I enclose check (or money order) for $2.50 ($2.00 if now a member in good standing of the A. Ph. A.) for which please send me POSTPAID, a copy of the 1954-55 Manual of Antibiotics.

Name.....

Address.....

.....



A Preview of Advertising

In the January 1955 issue of the Practical Pharmacy Edition

(All references to actions and uses are taken from the statements of the producers of the products mentioned.)

▸ Featured by **Abbott Laboratories** are **SUR-BEX,** vitamin B complex tablets, *ironplus* **Iberol,** and *filmtab* **Erythrocin Stearate,** an enteric coated, oral antibiotic.

▸ The **Chesebrough Mfg. Co.** emphasizes the dependability of its **Vaseline** sterile petrolatum gauze dressings.

▸ The **Crystalab Deeminizer,** product of **Crystal Research Laboratories,** turns tap water into "purified water" in one operation, "at less than 1¢ per gallon."

▸ **Romilar,** reports **Hoffmann-La Roche,** is a new, *non-narcotic* cough specific, the effect of which is "equal—if not superior—to codeine."

▸ The confirmed value of its **Thantis Lozenges** is reemphasized by **Hynson, Westcott & Dunning.**

▸ **Achromycin** is described by **Lederle Laboratories** as the *original* and *leading* brand of tetracycline.

▸ **Ilotycin Drops** "with an unexcelled antibiotic spectrum" are the subject of the **Lilly** announcement.

▸ The following prescription specialties are listed by the **S. E. Massengill Company** in its insertion: **Adrenosem, Aminodrox, Gallogen, Livitamin, Massengill Powder, Obedrin, Salcedrox** and **Semhyten.**

▸ **Owens-Illinois** reviews pictorially those of its products that "made important ℞ container news in 1954."

▸ For wintertime coughs, **Parke, Davis & Company** suggests **Ambenyl Expectorant** for quick relief.

▸ Extensively detailed this month among physicians are **Pfizer's** *flavor-favorite* **Terramycin** oral suspension and **Tetracyn** oral suspension.

▸ Robitussin A-C, for coughs aggravated by an allergic component, and **Donnatal Extentabs**—for prolonged spasmolysis—are pointed up by the **A. H. Robins Co.**

▸ **Bonadoxin,** for nausea-free pregnancy comfort, is suggested by **Roerig** and, for all treatable anemias, **Roetinic.**

▸ For the relief of cold symptoms, **Schering** advocates **Coricidin** with penicillin.

▸ In two insertions, **Smith, Kline & French Laboratories** stresses the ready salability of **Benzedrex Inhalers** and emphasizes the wide diversity of **Thorazine's** activity.

▸ **E. R. Squibb & Sons** announces two new antibiotics: **Steclin** and **Mycostatin.**

▸ **The Upjohn Company** proclaims **Cheracol** as the "best-seller for 28 years!"

▸ The **Warner-Chilcott** announcement reflects new sizes and forms of its hypotensive agent, **Methium,** and **Peritrate,** prophylaxis against angina pectoris.

Look for details in the Practical Pharmacy Edition of This Journal—Out January 20

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLIV — *JANUARY, 1955* — NUMBER 1 — CONSECUTIVE NO. 2

Synthetic Riboflavin-5′-Phosphate*

By E. De RITTER, J. SCHEINER, F. W. JAHNS, L. DREKTER, and S. H. RUBIN

Further confirmation of the full biological activity of synthetic riboflavin-5′-phosphate (FMN), including rat microbiological assays and human availability studies are described. Properties in relation to their effect on fluorometric and microbiological methods are presented. Synthetic FMN has full activity on a molar basis for growth of *L. casei* in the riboflavin assay.

THEORELL (1, 2) described the isolation of a riboflavin monophosphoric acid ester by a reversible splitting of protein from the yellow enzyme of Warburg and Christian (3). Following the synthesis of riboflavin by Karrer, *et al.* (4), and by Kuhn, *et al.* (5), a phosphate derivative of the vitamin was prepared by Kuhn and Rudy (6) and later shown (7) to combine with the protein group of the yellow enzyme in the same fashion as the natural riboflavin phosphate (flavin mononucleotide or FMN). By means of oxidation with periodic acid, the phosphate in FMN was shown by Karrer, *et al.* (8), to be in the 4′ or 5′-position in the riboflavin molecule. Forrest and Todd (9) prepared a synthetic product exhibiting the same paper chromatographic behavior as natural FMN. Periodate oxidation of the synthetic product indicated it to be the 5′-phosphate. A substance which also showed the same paper chromatographic properties was prepared by them in low yield via 5′-trityl,2′,3′,4′-triacetyl riboflavin according to the method of Kuhn, *et al.* (10), thereby providing additional confirmation that natural FMN is the 5′-phosphate.

FMN serves as the prosthetic group of a number of enzymes, as does flavin-adenine dinucleotide (FAD), which is formed from FMN by the addition of adenylic acid. The isolation of FAD has been described by Warburg and Christian (11) and its enzymatic synthesis from natural FMN and adenosine triphosphate by Schrecker and Kornberg (12). The biological importance of riboflavin-5′-phosphate is indicated by the fact that practically all of the riboflavin in many animal tissues occurs as the mononucleotide or to an even greater extent as FAD (13, 14).

Flexser and Farkas (15) have evolved a synthesis of FMN, which has made it available in large quantities. In addition to the free acid ester, both mono-diethanolamine and monosodium salts have been prepared. The biological activity of this synthetic FMN has been studied in various enzyme systems. For example, Kearney and Englard (16) found the synthetic product to be fully as active as natural FMN in its ability to activate the apo-enzyme of cytochrome *c* reductase of yeast according to the method of Haas, *et al.* (17). Riegel and Meyer (18) reported that synthetic FMN could replace a supernatant fraction of rat liver homogenate in tests of enzymatic inactivation of estradiol and diethylstilbestrol. Further confirmation of the full biological activity of the synthetic FMN is provided by the experiments described below, which include rat and microbiological assays and human availability studies. Properties of the FMN, some of which are of particular interest in relation to their effect on the usual fluorometric and microbiological methods for riboflavin assay, are described.

* Received Oct. 20, 1954 from the Nutrition Laboratories, Hoffmann-La Roche, Inc., Nutley, N. J.

RIBOFLAVIN ASSAYS

Rat Growth Tests.—Riboflavin assays by prophylactic and curative rat growth methods are given in Table I for two preparations of the monodiethanolamine salt of FMN. These assays were carried out according to a modification of the method of Street (19) with dosage by both oral and intramuscular routes. Twelve rats were used for each of the two levels of riboflavin standard and test material. A typical growth response in a prophylactic assay with oral dosage is shown in Fig. 1. Within the limits of error of the bioassay, these data indicate the FMN salt to have the same activity as riboflavin on a molar basis in all three types of bioassay.

TABLE I.—RAT-GROWTH ASSAYS OF MONODIETHANOLAMINE SALT OF RIBOFLAVIN-5′-PHOSPHATE (DIHYDRATE)

Lot No.	Type of Assay	Mode of Administration	Riboflavin Assay, % of Theoretical ± 2 S.E.
VI-101[a]	Prophylactic	Oral	95 ± 15
VI-116[b]	Curative	Oral	86 ± 15
VI-116	Curative	Intramuscular	101 ± 13

[a] Assayed 100% of theory fluorometrically and 99% microbiologically.
[b] Assayed 100% of theory fluorometrically and 98% microbiologically.

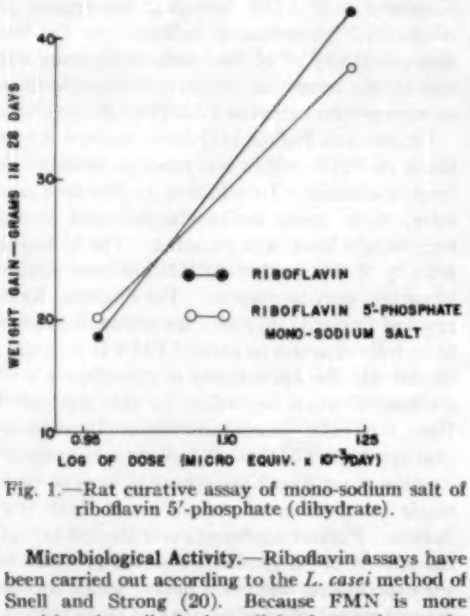


Fig. 1.—Rat curative assay of mono-sodium salt of riboflavin 5′-phosphate (dihydrate).

Microbiological Activity.—Riboflavin assays have been carried out according to the *L. casei* method of Snell and Strong (20). Because FMN is more sensitive than riboflavin to light destruction at the very low assay concentrations as detailed below, special precautions were taken to avoid exposure of test solutions. Operations were carried out as far as possible in a darkroom under Wratten Series OA filters and solutions were stored in amber or red flasks. Prior to autoclaving, the racks of assay tubes were placed in a covered metal box and kept therein until the forty-hour incubation was completed.

Synthetic preparations of FMN or its salts show the same activity for *L. casei* as riboflavin on a molar basis. To demonstrate this fact, a sample of mono-sodium salt (dihydrate) was assayed repeatedly to obtain a statistical average of both microbiological and fluorometric assays. In 13 tests of this preparation, which has a theoretical riboflavin content of 73%, the microbiological assays averaged 71.0% ± 2.4 (2 S.E.) and the fluorometric assays 71.0% ± 0.68 (2 S.E.). Prolonging the normal forty-hour incubation period in the microbiological assay to three, four, five, or seven days did not alter the relative activity of riboflavin and FMN.

Attempts by Theorell (2) to couple crude preparations of FMN with protein to form the yellow respiratory enzyme indicated that the position of the phosphate group in the molecule was of deciding importance. Linkage only at the 5′ or terminal position in the ribose side chain is essential for full micro-biological activity. Further phosphorylation in other positions of the ribose side chain may result in decreased activity for *L. casei* without affecting the fluorescence significantly. Hence, to assess the biological potency of FMN preparations, a biological or microbiological test is necessary. Such decreased activity upon esterification in other than the 5′-position has been reported by Furter, *et al.* (21), for mono-, di-, tri-, and tetra-succinates of riboflavin.

Fluorescence Measurements.—On a molar basis, the fluorescence of FMN prepared from natural sources has been reported by Bessey, *et al.* (13), to be equal to that of riboflavin. Similarly, the fluorescence of riboflavin in the synthetic product is unaffected by esterification in the 5′-position. Hence, estimation of the total percentage of riboflavin in preparations of FMN may be made conveniently by measurement of the fluorescence upon exposure to ultraviolet light. As noted above, however, the fluorescence may not indicate biological activity since it is not specific for the 5′-phosphate. Bessey, *et al.* (13), studied also the effect of *p*H between 1.3 and 6.6 on the fluorescence of riboflavin and natural FMN and found the latter to lose its fluorescence at a slightly higher *p*H than does the riboflavin. A similar study of the fluorescence of riboflavin and synthetic FMN between *p*H 2 and 11 is shown in Fig. 2. McIlvaine's phosphate citrate buffer was used from *p*H 2 to 8 with NaOH

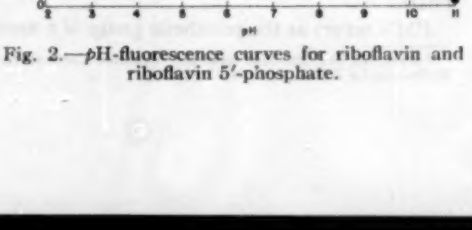


Fig. 2.—*p*H-fluorescence curves for riboflavin and riboflavin 5′-phosphate.

added to achieve *p*H levels above 8. The differences in fluorescence at the lower *p*H levels are very similar to those found by Bessey, *et al.* (13), for natural FMN. Between *p*H 4 and 7 both compounds show equal fluorescence on a molar basis. As the *p*H is increased above neutrality FMN again loses its fluorescence at a slightly higher *p*H than riboflavin.

Human Availability.—Relative urinary excretions of riboflavin after oral doses of riboflavin and FMN were determined according to the procedure described by Melnick, *et al.* (22), for testing physiological availability. Seven subjects participated in tests of both the free ester and its mono-diethanolamine salt (dihydrate), four taking in the first week a dose of 10 mg. of riboflavin and three an equivalent amount of the test substance. In the second week of the tests, the dose for each subject was reversed. Urinary excretions were measured fluorometrically by the double reduction method of Rubin, *et al.* (23), and microbiologically by the *L. casei* method noted above (20).

It was also of interest to determine whether FMN was excreted in the urine as such or as free riboflavin. For this purpose the benzyl alcohol extraction procedure of Burch, *et al.* (24), as applied to the macro assay of urine, was followed exactly except that all volumes were doubled to provide 15 ml. for fluorometric reading. By this method, riboflavin is extracted almost quantitatively by the benzyl alcohol as compared to only a few per cent of FMN or its salt. A summary of the excretion data is given in Table II. If the term "availability" is taken to denote the relative urinary excretion after FMN and riboflavin dosage, it is evident from the data in Table II that both the free ester and its diethanolamine salt are 100 per cent available. The fact that the average excretions determined by the benzyl alcohol technique are not significantly different from those found by the direct fluorometric and microbiological methods indicates that the excretion after both riboflavin and FMN ingestion, is largely, if not entirely in the form of free riboflavin. The sensitivity of this method is not sufficient to prove or disprove the presence of a small percentage of FMN. That the excretion in the form of FAD is negligible has been shown by Klein and Kohn (25) by means of the alanine test of Warburg and Christian (11).

PROPERTIES OF SYNTHETIC FMN

Sensitivity to U. V. Light.—While measuring the fluorescence of solutions of FMN at concentrations of about 0.15 μg. per ml., it was observed that the destruction of fluorescence on exposure to the ultraviolet light of the fluorophotometer was more rapid than in comparable solutions of riboflavin. This is illustrated in Fig. 3, which shows the relative rates of destruction of FMN and riboflavin during ten minutes exposure in the cuvettes of a Pfaltz and Bauer Model B Fluorophotometer with a Type H-3 mercury lamp, a primary filter combination having a peak at about 430 mμ, and a secondary filter to eliminate wave lengths below 520 mμ. Solutions of both compounds were compared in 0.015 *M* acetate buffer at *p*H about 5.9 and in 0.113 *M* phosphate + 0.033 *M* citrate buffer at *p*H about 6.0. In both buffers the losses of FMN are greater than those of riboflavin, the difference in rate of destruction being

TABLE II.—PHYSIOLOGICAL AVAILABILITY TO HUMANS OF RIBOFLAVIN-5′-PHOSPHATE, FREE ESTER AND MONO-DIETHANOLAMINE SALT (DIHYDRATE)

	% Recovery in Urine of Test Dose (equiv. to 10 mg. of riboflavin)					
	Fluorometric Assay				Microbiological Assay with *L. casei*	
	Direct Method (23)		In Benzyl Alcohol (24)			
Subject	Riboflavin	FMN Free ester	Riboflavin	FMN Free ester	Riboflavin	FMN Free ester
EM	58	70	60	63	65	60
IK	58	66	40	60	60	62
EDR	47	65	53	52	58	58
JS	71	68	65	65	75	69
WK	59	45	37	49	59	43
RW	42	42	44	35	44	41
MW	55	60	55	59	59	69
Av.	56	59	51	55	60	57
		Mono-diethanol-amine salt		Mono-diethanol-amine salt		Mono-diethanol-amine salt
EM	52	61	50	56	49	57
BM	65	58	63	65	59	60
NC	51	60	55	50	57	57
EDR	46	61	40	56	50	63
ES	53	56	54	58	54	61
RW	41	45	42	51	38	48
FWJ	47	48	40	48	46	53
Av.	51	56	49	55	50	57

	% Availability ± S.E.		
	Fluorometrically		
	In Aqueous Soln.	In Benzyl Alcohol	Micro-biologically
FMN, free ester	105 ± 10.0	108 ± 11.6	95 ± 8.9
FMN, mono-diethanolamine salt (dihydrate)	110 ± 7.9	112 ± 8.9	114 ± 7.2

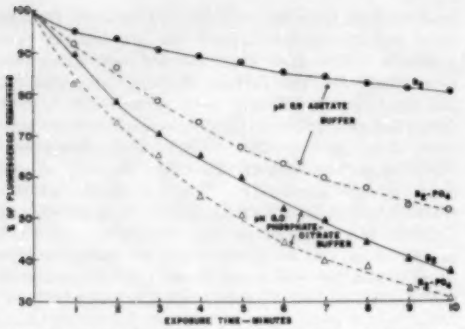


Fig. 3.—Relative rate of destruction of riboflavin and riboflavin-5′-phosphate by UV light.

most marked at the beginning of exposure. Higher losses were noted in the phosphate-citrate buffer, but the difference between FMN and riboflavin is greater in the acetate buffer. Because of this greater sensitivity of FMN in very dilute solutions, care must be taken to avoid exposure to light. The use of a photocell with a rapid response is desirable for precise measurements of fluorescence. The fluorescence may also be affected by the quality of the distilled water used for dilution. The use of ion-exchange purified water or prior addition of a chelating agent to the water will eliminate such difficulty. These special precautions are essential only at the very low concentrations involved in the analysis and not at normal pharmaceutical levels, which are usually at least a thousand-fold higher.

Enzymatic Hydrolysis.—The liberation of free riboflavin from FMN by treatment with an acid phosphatase (Clarase) was reported by Bessey, *et al.* (13). The rate of such hydrolysis of synthetic FMN (Fig. 4) has been determined by measurement

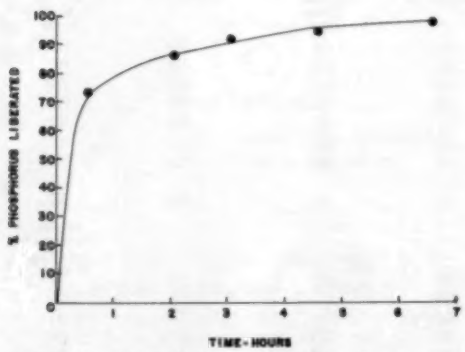


Fig. 4.—Liberation of phosphate from mono sodium salt of riboflavin-5′-phosphate by enzyme (Clarase) treatment at 45° and *p*H 4.5.

of the free phosphate produced, using a modification of the method of Lohmann and Jendrassik (26), which distinguishes between free and organically bound phosphate. Eight-milliliter aliquots containing 4 mg. of mono-sodium salt of FMN (dihydrate) in 0.05 *M* acetate buffer at *p*H 4.5 were digested at 45° with 10 mg. of Clarase for five-tenths to six and five-tenths hours, after which enzyme action was stopped by steaming for ten minutes and free phosphate determined. Parallel blank solutions were set up without FMN to correct for phosphate contributed by the enzyme. It is evident (Fig. 4) that the enzyme splitting of FMN is rapid, over 70% of the phosphate being liberated in the first half hour of incubation.

Acid Hydrolysis.—The directions for fluorometric and microbiological riboflavin assays by the U. S. P. method (27) call for autoclaving samples in 0.1 *N* HCl for thirty minutes at 121–123° in order to dissolve the riboflavin. In assaying riboflavin 5′-phosphate, application of this acid hydrolysis has been found to cause a 15% loss of microbiological riboflavin activity. Under the same conditions the fluorescence is decreased by only 2–3%. For this reason acid hydrolysis should be avoided, particularly in microbiological assays. Because of the high solubility of the phosphate in water, no difficulty is encountered in preparing assay solutions without such hydrolysis. Simple shaking, or in the case of tablets, blending in water with adequate protection from actinic light is sufficient.

SUMMARY

1. Synthetic ribroflavin-5′-phosphate (FMN) is as active on a molar basis as riboflavin in promoting growth of weanling rats in curative assays with oral or intramuscular dosage or in a prophylactic test with oral dosage.

2. Synthetic FMN has full activity on a molar basis for growth of *L. casei* in the riboflavin assay. Since full microbiological activity is achieved only with substitution in the 5′-position, the microbiological assay is the method of choice for determining the purity of FMN preparations rather than the fluorometric assay which is less sensitive to phosphorylation at other points in the ribose chain.

3. Comparison of urinary excretion after oral doses of riboflavin and FMN shows the latter to be completely available to humans. Excretion of both substances is largely, if not entirely, in the form of free riboflavin.

4. The more rapid destruction by light of FMN than of riboflavin at the very low concentrations used in fluorometric and microbiological assays necessitates careful protection of such solutions from light exposure. Acid hydrolysis, which is also destructive, should not be applied in the assay of FMN.

5. The phosphate group is split rapidly from FMN by enzymatic hydrolysis with Clarase at *p*H 4.5 and 45°.

REFERENCES

(1) Theorell, H., *Biochem. Z.*, **275**, 344(1935).
(2) Theorell, H., *ibid.*, **278**, 263(1935).
(3) Warburg, O., and Christian, W., *ibid.*, **254**, 438 (1932).
(4) Karrer, P., Schoepp, K., and Benz, F., *Helv. Chim.*

Acta, **18**, 426(1935).
(5) Kuhn, R. Reinemund, K., Weygand, F., and Stroebele, R., *Ber.*, **68**, 1765(1935).
(6) Kuhn, R., and Rudy, H., *ibid.*, **68**, 383(1935).
(7) Kuhn, R., and Rudy, H., *ibid.*, **69**, 1974(1935).
(8) Karrer, P., Frei, P., and Meerwein, H., *Helv. Chim. Acta*, **20**, 79(1937).
(9) Forrest, H. S., and Todd, A. R., *J. Chem. Soc.*, **1950**, 3295.
(10) Kuhn, R., Rudy, H., and Weygand, F., *Ber.*, **69**, 1543 (1936).
(11) Warburg, O., and Christian, W., *Biochem. Z.*, **298**, 150(1938).
(12) Schrecker, A. W., and Kornberg, A., *J. Biol. Chem.*, **182**, 795(1950).
(13) Bessey, O. A., Lowry, O. H., and Love, R. H., *ibid.*, **180**, 755(1949).
(14) Crammer, J. L., *Nature*, **161**, 349(1948).
(15) Flexser, L. A., and Farkas, W. G., Abstracts, XIIth International Congress of Pure and Applied Chemistry, New York, 1951, p. 71. Also U. S. patents 2,604,470; 2,610,176, -7, -8, -9.
(16) Kearney, E. B., and Englard, S., *J. Biol. Chem.*, **193**, 821(1951).
(17) Haas, E., Horecker, B. L., and Hogness, T. R., *ibid.*, **136**, 747(1940).
(18) Riegel, I. L., and Meyer, R. K., *Proc. Soc. Exptl. Biol. Med.*, **80**, 617(1952).
(19) Street, H. R., *J. Nutrition*, **22**, 399(1941).
(20) Snell, E. E., and Strong, F. M., *Ind. Eng. Chem., Anal. Ed.*, **11**, 346(1939).
(21) Furter, M. F., Haas, G. J., and Rubin, S. H., *J. Biol. Chem.*, **160**, 293(1945).
(22) Melnick, D., Hochberg, M., and Oser, B. L., *J. Nutrition*, **30**, 67(1945).
(23) Rubin, S. H., De Ritter, E., Schumann, R. L., and Bauernfeind, J. C., *Ind. Eng. Chem., Anal. Ed.*, **17**, 136 (1945).
(24) Burch, H. B., Bessey, O. A., and Lowry, O. H., *J. Biol. Chem.*, **175**, 457(1948).
(25) Klein, J. R., and Kohn, H. I., *ibid.*, **136**, 177(1940).
(26) Lohmann, K., and Jendrassik, L., *Biochem. Z.*, **178**, 419(1926).
(27) U. S. Pharmacopeia, 14th Rev., Mack Publishing Co., Easton, Pa., **1950**, p. 750.

The Relative Efficacy of Various Ointment Bases as Antiseptic Vehicles*

By CHARLES K. KOLSTAD and C. O. LEE†

This study indicates that the Beeler base, methyl cellulose base, and polyethylene glycol ointment are more efficient vehicles for ammoniated mercury, boric acid, and yellow mercuric oxide than the official white ointment. The Beeler base and polyethylene glycol ointment proved to be better vehicles for iodine than the official yellow ointment. Petrolatum-rose water ointment is a more efficient vehicle for ammoniated mercury and for yellow mercuric oxide than the official white ointment despite the incompatibility between the yellow mercuric oxide and the base.

IN THE USE OF OINTMENTS for the administration of antiseptics, two questions arise. First, which ointment base is best suited for the medicinals prescribed? Second, will a good antiseptic act as such, irrespective of the base in which it is incorporated?

So far as we know, no one has, as yet, proposed the base most suitable for efficient therapeutic action of the medicaments commonly used in the form of ointments. Investigators differ regarding the type of base to use in most instances. Some favor the use of bases which contain a high percentage of water (1, 2). Others maintain that the greasy bases are more efficient (3–6). Still others feel that one type of base is as good as another (7, 8). The ointments that are official in both the Pharmacopeia and the National Formulary are, for the most part, prepared with greasy bases.

A RÉSUMÉ OF PREVIOUS WORK

In 1929, Reddish and Wales (9) tested the antiseptic properties of 26 official ointments, 12 in the U. S. Pharmacopoeia X, and 14 in the National Formulary V. Of these, 5 of the U. S. P. and 6 of the N. F. ointments proved to be antiseptic when tested by the FDA method. Among those preparations which showed no antiseptic action was the official 2% phenol ointment. This observation, concerning phenol ointment, was confirmed by later workers (2, 10, 11).

Comprehensive studies of the action of antiseptics in a variety of ointment bases were made by Prout and Strickland (3) in 1937, Gershenfeld and Brillhart (1) in 1939, and Foley and Lee (8) in 1942. In 1945, Fiero and Loomis (12) and Neuroth and Lee (13) investigated the activity of a single antiseptic in a variety of bases. Hart and Huyck (14), in 1948 tested a series of quaternary ammonium compounds in several bases in an effort to determine the base most suitable as a vehicle for them.

The findings of these investigators substantiated the idea of Reddish, that no single base is wholly satisfactory as the vehicle for all antiseptics. In view of this fact, and for other reasons, this study was made in an effort to determine the relative antiseptic efficacy of four well-known chemicals using five of the newer ointment bases.

* Received July 21, 1954 from the Research Laboratories of the Purdue University School of Pharmacy, Lafayette, Ind.
† Present address: Ohio Northern University, College of Pharmacy, Ada.

EXPERIMENTAL

The four chemicals used were iodine, boric acid, ammoniated mercury, and yellow mercuric oxide. The official ointments of these chemicals were used as the standards of comparison for the ointments prepared from the newer bases.

The five newer bases were: Beeler Base, Jelene 50W, Methyl Cellulose Base, Petrolatum Rose Water Ointment U. S. P., Polyethylene Glycol Ointment U. S. P.

The formulas for the official bases are easily available and need not be given here. Those for the three unofficial bases are as follows:

Beeler Base (15)		Methyl Cellulose Base	
Cetyl alcohol	15 Gm.	Methyl cellulose	2 Gm.
White wax	1 Gm.	Glycerin	10 Gm.
Propylene glycol	10 Gm.	Water	88 Gm.
Sodium lauryl sulfate	2 Gm.		
Water	72 Gm.		

Jelene ointment base is a product of the Research Products Corporation, Madison 10, Wis. It is described as a combination of mineral oils and heavy hydrocarbon waxes (16). Jelene 50W may be obtained from E. R. Squibb and Son.

METHOD OF TESTING

The method of evaluating the antiseptic properties of the ointments in this study was that of the Food and Drug Administration as described by Ruehle and Brewer (17). The test organism was *Staphylococcus aureus* obtained from the bacteriology department of Purdue University.

A modification of the agar cup plate method which was used is described here. Ten ml. of plain agar were pipetted into a sterile Petri plate and allowed to harden. A penicillin assay cup was then placed upright on the hard agar and 20 ml. of seeded agar pipetted around it. When the latter had solidified, the cup was removed leaving a hole in the plate.

The ointments to be tested had been put in 1-oz. tubes and weighed. Test samples were taken by holding the tube above the hole in the agar and carefully extruding ointment into the cup until it was filled. The ribbon of ointment was severed with a warm spatula and the tube reweighed. The amount of ointment deposited in the cup was easily calculated.

The plate was placed in an incubator at a temperature of 37° for twenty-four hours. The clear zone produced by the antiseptic was measured with a Fisher-Lilly Antibiotic Zone Reader. Each zone was measured from one point; then the plate was rotated through 90 degrees and another measurement made.

At the outset of this study, ten tests of each ointment were made. This number was reduced when it was found that six plates were sufficient to insure accurate and valid results.

Many of the nonofficial ointments produced larger zones of inhibition than did the official ointments. The nonofficial ointments were then progressively reduced in medicinal strength to determine the concentration that was equivalent to the official ointment in inhibiting the growth of microorganisms. The results obtained in this study are summarized in Table I. Each figure given represents the average millimeter width of the clear zones of not less than 6 plates.

DISCUSSION

The Beeler base proved to be the most efficient vehicle for iodine. The weakest concentration (0.25%) that we tested gave an inhibition zone greater than the official 4% ointment. The zones

TABLE I.—COMPOSITE TABLE OF RESULTS

Ointments, Gm. per 100	Width of Clear Zones in Mm. of Ointments Tested				
	Official Preparation	Beeler Base	Jelene 50W	Methyl Cellulose	Rose Water Ointment	Polyethylene Glycol
Iodine						
4.00 Gm.	3.7	17.8	14.9	[a]	[a]	11.5
2.00 Gm.		15.1	4.0			12.5
0.10 Gm.		13.1	3.0			11.2
0.50 Gm.		8.4				7.6
0.25 Gm.		4.3				3.7
Yellow Mercuric Oxide						
1.000 Gm.	5.0	15.5	4.6	10.2	9.7	14.5
0.500 Gm.		12.1	4.0	10.0	8.1	12.4
0.250 Gm.		10.9	3.4	10.4	8.3	11.6
0.120 Gm.		9.6		9.5	7.2	10.6
0.060 Gm.		8.6		8.6	5.9	9.8
0.030 Gm.		5.0		5.9	1.9	6.1
0.015 Gm.		4.7		4.3		3.6
Ammoniated Mercury						
5.00 Gm.	6.2	11.1	8.2	10.7	8.5	13.4
2.50 Gm.		5.5	5.0	9.2	7.2	10.4
1.25 Gm.		1.9	3.8	6.0	4.9	5.0
Boric Acid						
10.00 Gm.	0.0	6.9	0.0	8.6	0.0	8.0
5.00 Gm.		6.4		8.1		4.4
2.50 Gm.		0.0		3.4		2.1

[a] Incompatible.

produced by iodine in all of its dilutions in the Beeler base were larger than the zones produced by any of the other bases.

Yellow mercuric oxide, in all of its strengths, was released more effectively from the polyethylene glycol base than from the other bases. This medicament in a 1% strength gave a wider zone in the Beeler base than it did in the polyethylene glycol base, but in most lesser strengths the glycol preparation gave better results.

Ammoniated mercury in 5% concentration in the polyethylene glycol base was an effective inhibitory agent but in a concentration of 1.25% it was more active in the methyl cellulose base than in the other bases.

Boric acid, in the three per cent strengths tested, exhibited its greatest activity in the methyl cellulose base.

The results of these tests would seem to indicate that the presence of water in a base is not an essential requisite for antiseptic action. The polyethylene glycol base, which contained no water, produced zones equal to or larger than the bases which contained water. Ointments in the methyl cellulose base, which contained the highest percentage of water, were not always the most efficient inhibitors of the growth of the microorganisms.

It should be recognized, perhaps, that there are many factors that influence the release of a medicament from an ointment base. The polyethylene glycol base, although containing no water, is readily soluble and should aid the rapid diffusion of water-soluble medicaments. On the other hand, the colloidal nature of the hydrophilic methyl cellulose base may inhibit or delay the release of medicaments. This is to suggest that we need to learn much more about the physical properties of bases than we now know. Perhaps the composition of ointment bases is of lesser importance than their physical properties. At least the idea might be worth investigating.

REFERENCES

(1) Gershenfeld, L., and Brillhart, R. E., *Am. J. Pharm.*, **111**, 430(1939).
(2) Gershenfeld, L., and Miller, R. E., *ibid.*, **105**, 186 (1933).
(3) Prout, W. A., and Strickland, M., THIS JOURNAL, **26**, 730(1937).
(4) Baron, B., and DeKay, H. G., *ibid.*, **32**, 294(1943).
(5) Busse, L. W., *ibid.*, *Pract. Pharm. Ed.*, **4**, 314(1943).
(6) Halpern, A., and Hartwell, L., *Am. J. Pharm.*, **120**, 386(1948).
(7) Gibson, A. J., Parker, H. E., and Almus, A., THIS JOURNAL, **30**, 196(1941).
(8) Foley, E., and Lee, C. O., *ibid.*, **31**, 105(1942).
(9) Reddish, G. F., and Wales, H., *ibid.*, **18**, 576(1929).
(10) Husa, W. J., and Radin, J. M., *ibid.*, **21**, 861 (1932).
(11) Craw, R. O., and Lee, C. O., *Pharm. Arch.*, **9**, 1(1938).
(12) Piero, G. W., and Loomis, T. A., THIS JOURNAL, **34**, 218(1945).
(13) Neuroth, M. L., Lee, C. O., Christian, J. E., and Jenkins, G. L., *ibid.*, **35**, 321(1946).
(14) Hart, F., and Huyck, C. L., *ibid.*, **37**, 272(1948).
(15) Beeler, E. C., *Bull. Natl. Formulary Comm.*, **11**, 27 (1943).
(16) Foster, S., *et al.*, THIS JOURNAL, **40**, 123(1951).
(17) Ruehle, G. L. A., and Brewer, G. M., "United States Food and Drug Administration Methods for Testing Antiseptics and Disinfectants," U. S. Dept. Agriculture Circ., 198. 1931.

An Evaluation of the Stability of Buffered Erythromycin Ointments*

By HAROLD SHEINAUS† and C. O. LEE‡

The stability of 1 per cent erythromycin in buffered bases of the Beeler formula has been tested *in vitro* by plating against M. *pyogenes* var. *aureus*, after varying storage periods at room temperature. The results indicate the advisability, under the given test conditions, of using a buffered base such as to give an erythromycin ointment with a *p*H of approximately 8.6, for maximum stability.

ERYTHROMYCIN, one of the newer antibiotics obtained from *Streptomyces erythreus*, is effective against a wide range of microorganisms, as well as certain rickettsiae and certain large viruses (1). Its antibacterial spectrum is closely related to that of penicillin, and at present it is one of the antibiotics of choice in the treatment of infections attributed to strains of staphylococci resistant to other antibiotics (2).

The instability of erythromycin in aqueous solution would seem to warrant investigation as to the stabilizing effect of buffering it at various *p*H levels. McGuire, *et al.* (3), state that erythromycin readily forms salts with acids, but preliminary tests by us indicated that it is inactivated to a large degree at an acid *p*H. Heilman and co-workers (4) found, in cup-plate experiments, that at a concentration of 2μg./ml. in saline, the activity of erythromycin was markedly reduced as the *p*H of the solution was varied from 8 to 6.4.

In this study we were primarily concerned with the stability of this antibiotic in ointments prepared for extemporaneous use. For that reason, water-holding bases of the O/W type, buffered at various alkaline levels, were used.

EXPERIMENTAL AND RESULTS

A simple O/W emulsion base, the Beeler base (5), was chosen as the test vehicle. The formula is as follows:

* Received July 21, 1954, from the Research Laboratories of the Purdue University School of Pharmacy, Lafayette, Ind.
† Present address: Carroll Durham Smith Pharmacal Co., New Brunswick, N. J.
‡ Present address: Ohio Northern University, College of Pharmacy, Ada.

Cetyl alcohol	15.0
White wax	1.0
Propylene glycol	10.0
Sodium lauryl sulfate	2.0
Distilled water	72.0

Six modifications of this base were prepared using U. S. P. XIV Clark and Lubs boric acid-KCl-NaOH buffer solutions of six different *p*H values, in place of distilled water. The regular Beeler base, unmodified, served as the control.

Erythromycin[1] (963 μg/mg.) was levigated with a small, controlled amount of alcohol and incorporated in each of the bases in a concentration of 10 mg./Gm.

Samples of the buffer solutions and the buffered bases prepared with these solutions, to which a proportional quantity of alcohol was also added, were retained and stored in collapsible tin tubes and in opal glass ointment jars for observation of *p*H changes which might occur over the test period. The buffered erythromycin ointments were also stored in collapsible tin tubes. All containers were stored at a room temperature of 27° ± 2°.

pH Determinations.—The *p*H values of (*a*) the buffer solutions and (*b*) the buffered bases in both types of container, and of (*c*) the erythromycin ointments, were measured at several intervals during the sixteen-week test period, using a Beckman *p*H meter, model N. The *p*H determinations of the ointments in tubes were carried out by extruding a sufficient amount of ointment, and carefully covering both electrodes, with an ointment bridge between. Reproducible, nonfluctuating readings were obtained by this method. Data for these determinations are summarized in Tables I, II, and III.

TABLE III.—*p*H OF ERYTHROMYCIN OINTMENTS

Ointment	Initial *p*H value	After 4 weeks	After 10 weeks	After 16 weeks
A	7.5	7.7	7.7	7.7
B	7.7	7.9	8.0	8.0
C	8.2	8.25	8.3	8.3
D	8.6	8.7	8.75	8.7
E	9.0	9.2	9.25	9.3
F	11.1	9.6	9.5	9.35
Control	9.0	9.1	9.1	9.1

bases were, in turn, used to prepare the erythromycin ointments lettered correspondingly in Table III.

The *p*H values of the buffer solutions themselves, as seen from Table I, remained fairly constant in glass, but varied considerably in the tubes at higher *p*H values. Solutions **E** and **F** stored in the tubes developed a brown sediment, apparently due to chemical reaction with the tube. In tube **F**, the reaction was sufficiently corrosive to cause the solution to eat through the tube at the level of the solution-air interface. The tubes were of the ordinary variety used to dispense extemporaneously prepared ointments.

The bases prepared with the buffer solutions showed some instability in tubes, but were relatively stable in glass. This can be seen in Table II. In particular, base **F** in the tube dropped in value considerably. However, neither base **E** nor base **F** showed any evidence of discoloration, as was evidenced by the buffer solutions **E** and **F** alone.

The buffered erythromycin ointments which were stored in tubes only, showed slight changes in *p*H,

TABLE I.—*p*H OF BUFFER SOLUTIONS

Solution[a]	Initial Value	4 Weeks		10 Weeks		16 Weeks	
		Tube	Jar	Tube	Jar	Tube	Jar
A	7.3	7.3	7.2	7.85	7.3	8.1	7.3
B	8.15	8.05	8.1	8.15	8.1	8.15	8.1
C	8.9	8.7	8.8	8.75	8.85	8.75	8.9
D	9.4	9.1	9.3	9.1	9.4	9.1	9.4
E	10.2	9.3[c]	10.2	9.1[c]	9.9	9.1[c]	9.7
F	11.9	9.5[c]	11.8	8.2[c]	11.9	8.7[c]	10.9
Control[b]	5.9	8.4	6.0	7.4	6.1	7.7	6.1

[a] Clark and Lubs Buffer Mixtures.
[b] Distilled water.
[c] Developed a brown sediment in tube.

TABLE II.—*p*H OF BASES PREPARED WITH BUFFER SOLUTIONS

Ointment Base	Initial Value	4 Weeks		10 Weeks		16 Weeks	
		Tube	Jar	Tube	Jar	Tube	Jar
A	6.2	7.15	6.5	7.1	6.5	7.05	6.55
B	6.9	7.2	6.9	7.3	7.0	7.2	7.05
C	7.7	7.8	7.6	7.9	7.65	7.9	7.7
D	8.2	8.4	8.15	8.5	8.2	8.4	8.2
E	8.9	9.0	8.8	9.2	8.9	9.1	8.9
F	11.6	10.6	11.55	10.4	11.75	10.3	11.6
Control	7.2	9.3	9.2	8.8	9.3	8.4	9.3

The six buffer solutions mentioned above are designated by letters **A** to **F** in Table I. Distilled water served as the control. Solutions **A** to **F**, and control solution, were used to prepare bases **A** to **F** and control base, respectively, in Table II. These

[1] Supplied by Abbott Laboratories, North Chicago, Ill.

gradually rising one- or two-tenths of a *p*H unit over the test period. Here again, however, ointment **F** showed a large drop.

Inhibitory Activity of Erythromycin Ointments.—At various intervals during the sixteen-week test period, the buffered erythromycin ointments were

TABLE IV.—ZONES OF INHIBITION (DIAMETERS IN MM.)

Ointment	Initial Value	Weeks After Preparation 2	4	6	9	11	13	16
A	19	19	17	17	15	14.5	14	13
B	19	19	17	17	15	17	16.5	16
C	20	20	17	18	16.5	17.5	17	16.5
D	21	20	18	20	18.5	18.5	18	18
E	19	21	18.5	18	17.5	19.5	19.5	19.5
F	19	16	14	17	16.5	17.5	17	16.5
Control	18	19	16	17	17.5	17	17	16.5

plated against M. *pyogenes* var. *aureus* (FDA 209 P), using 21 ml. base agar[2] and 4 ml. seed agar[3] containing 0.08 ml. inoculated nutrient broth[4] which had been incubated at 37° for twenty-four hours. The plating was carried out by pressing the ointment from the tubes into glass cylinders 10 mm. long, having a 4 mm. internal and a 6-mm. external diameter. The tubes were dropped in place from a height of about one centimeter and the plates incubated at 37° for eighteen hours.

The plating tests indicated that erythromycin ointments **D** and **E** were the most active *in vitro*. However, because of the sediment developed by buffer solution **E** in the tube, it was felt that ointment **D** was to be preferred. This would indicate that the most stable ointment, under the conditions of these tests, would be one prepared with a buffer solution of *p*H approximately 9.4. This would give an ointment base with a *p*H of approximately 8.2, and a 1% ointment with a *p*H of approximately 8.6.

A note of interest might be included here. A ring of augmented growth around the inhibition zone sometimes occurs in the plating of certain antibiotics (6). Dufrenoy and Pratt (7) have stated that this phenomenon may be due to the stimulating effect of subbacteriostatic levels of the antibiotic. In preliminary observations in the case of erythromycin, we noted that after the 18-hour incubation period, no such ring appeared. After ninety-six hours of incubation, however, an extremely wide zone of augmented growth was present. This zone was approximately 8 mm. wide. Oxytetracycline and chlortetracycline in the same strength, in the same base, both showed a 1- to 2-mm. zone of augmented growth which appeared at eighteen hours and remained the same in size through ninety-six hours. Erythromycin could possibly be growth-stimulating over a wider range of concentrations than the other two antibiotics. No further observations along these lines were made.

[2] Bacto Penassay Base Agar, Difco Laboratories, Detroit, Mich.
[3] Seed Agar, Baltimore Biological Laboratories, Baltimore, Md.
[4] Bacto Penassay Broth, Difco Laboratories, Detroit, Mich.

REFERENCES

(1) Powell, H. M., *et al.*, *Antibiotics & Chemotherapy*, **3**, 165(1953).
(2) Grigsby, M. E., Johnson, J. B., and Simmons, G. W., *ibid.*, **3**, 1029(1953).
(3) McGuire, J. M., *et al.*, *ibid.*, **2**, 281(1952).
(4) Heilman, F. R., *et al.*, *Proc. Staff Meetings Mayo Clinic*, **27**, 285(1952).
(5) Beeler, E. C., *Bull. Natl. Formulary Comm.*, **11**, 27 (1943).
(6) Florey, H. W., *et al.*, "Antibiotics," Vol. 1, Oxford University Press, London, 1949, p. 141.
(7) Dufrenoy, J., and Pratt, R., *J. Bact.*, **53**, 657(1947).

Beta-Phenylethyl Alcohol as a Preservative for Ophthalmic Solutions*

By I. W. GROTE and MARIBELLE WOODS

β-Phenylethyl alcohol appears to be non-irritating to the rabbit eye when used in a three tenths per cent aqueous solution and the pure chemical has a low oral toxicity in rats.

PHENYLETHYL alcohol has recently been suggested as a preservative for ophthalmic solutions by Brewer, *et al.* (1), Lilley and Brewer (2), Goldstein and Ryan (3) because of its excellent antiseptic action against air-borne organisms. Several pharmacological and physical properties pertinent to such a use seemed to need further investigation. These include the possible irritating effect on the eye, the effect of the chemical on the activity of the natural antiseptic, of lysozyme, normally present in the tear and a more accurate determination of the toxicity of the alcohol if solutions were ingested by children. It also seemed of importance to determine the surface tension of the 0.3 per cent solution proposed for ophthalmic use.

* Received July 22, 1954 from the University of Chattanooga, Chattanooga, Tenn.
Our thanks are due to the Murine Company, Chicago, Ill., for aid in this work.

EXPERIMENTAL

Effect on Rabbit Eyes.—Two drops of a 0.3% solution (by volume) β-phenylethyl alcohol in 0.85% sterile saline were dropped in the left eye of two rabbits three times daily at 8:00 a. m., 12 noon, and 4:00 p. m. five days per week, for two weeks. The right eye of each rabbit served as the control. Following thirty applications of the 0.3% phenylethyl alcohol within a twelve day period to the left eye of two rabbits there was no redness, discharge, or indication of irritation.

Effect on Lysozyme.—Almost all of the saprophytic bacteria of the air (the sarcinae) are susceptible to the antiseptic action of the tears. This is due in a large measure to the lysozyme normally present in the tears. The effect on *Sarcina lutea* of a solution of lysozyme in sterile saline with and without phenylethyl alcohol was accordingly determined. Solution A was prepared to contain 1 mg./cc. crystalline lysozyme (Armour) and 0.3% (by volume) phenylethyl alcohol in 0.85% sterile saline. Solution B was prepared to contain 1 mg./cc. crystalline lysozyme (Armour) in 0.85% sterile saline. After Solutions A and B had stood for three hours at room temperature a 1:10, 1:100, and 1:1,000 dilution of each was made with sterile saline. Using small sterile test tubes 1 cc. of each of the dilutions of lysozyme with and without phenylethyl alcohol was placed in a separate sterile tube. To each of these tubes was added 1 cc. sterile saline and 1 cc. of a 24-hour culture *Sarcina lutea* in nutrient broth. Following a 24-hour incubation period at room temperature streak plates (nutrient agar) were made and the presence or absence of growth of *Sarcina lutea* on the plates noted. The contents of the tubes and the results are shown in Table I.

TABLE I.—THE EFFECT OF PHENYLETHYL ALCOHOL ON LYSOZYME

Tube No.	Saline, cc.	*Sarcina lutea* Culture, cc.	Test Solution, cc.	Agar Plate Growth, 24 hours
1	1	1	1 A	−
2	1	1	1 A/10	−
3	1	1	1 A/100	−
4	1	1	1 A/1,000	+
5	1	1	1 B	−
6	1	1	1 B/10	−
7	1	1	1 B/100	−
8	1	1	1 B/1,000	+
9	2	1	0	+

+ = abundant or maximum growth; − = no growth.
Solution A—1 mg./cc. crystalline lysozyme and 0.3% phenylethyl alcohol in 0.85% sterile saline.
Solution B—1 mg./cc. crystalline lysozyme in 0.85% sterile saline.

The bactericidal activity of lysozyme was not destroyed or changed by the presence of 0.3% phenylethyl alcohol for a period of three hours, when tested against *Sarcina lutea*. Lysozyme is a potent antiseptic against *Sarcina lutea* as is shown by the fact that it kills this organism at a dilution of 1:300,000.

Acute Oral Toxicity.—Undiluted beta-phenylethyl alcohol was given orally by stomach tube to female rats weighing 125–180 Gm. The animals were observed for twenty-four hours and the fatalities occurring within that time were recorded. The results are given in Table II. The LD_{50} was found to be 3.1 cc./Kg. female rat. The rats which survived the test period were permitted food and water *ad lib.* and were observed for three days for signs of delayed toxicity. All animals surviving the 24-hour test period were alive seventy-two hours later except one rat on 3 cc./Kg. It died twenty-five hours after the drug was administered. Previous workers Hjort and Eagen (4) and Macht (5) have reported on the acute toxicity of phenylethyl alcohol but their results have not been given in a quantitative way.

TABLE II.—ACUTE ORAL TOXICITY OF PHENYLETHYL ALCOHOL TO FEMALE RATS
24-Hour Test Period

Cc./Kg.	Number Rats	Number Alive	Number Dead	Per Cent Dead
4.0	5	1	4	80
3.0	5	3	2	40
2.0	5	4	1	20
1.0	5	5	0	0

LD_{50} = 3.1 cc./Kg.

Surface Tension.—Three tenths and 0.5% solutions (by volume) phenylethyl alcohol were prepared in demineralized water. The apparent surface tension of the water and the two solutions was determined on a Cenco-du-Noüy Tensiometer. Ten readings were made for each of the solutions and the average value given as the apparent surface tension. The true surface tension was obtained by multiplying the apparent surface tension by a correction factor F, determined according to Zuidema and Waters (6). The temperature of the room was 25° and the temperature of the test solution was 26°. The results are given in Table III. The true surface tension for demineralized water was 71.4 dynes per cm., for 0.3% phenylethyl alcohol it was 60.5 dynes per cm., and for 0.5% phenylethyl alcohol it was 56.4 dynes per cm.

TABLE III.—SURFACE TENSION OF PHENYLETHYL ALCOHOL IN 0.3% AND 0.5% SOLUTION
Apparent Surface Tension Readings

	Water, dynes per cm.	0.3% Phenylethyl Alcohol, dynes per cm.	0.5% Phenylethyl Alcohol, dynes per cm.
	76.3	64.9	60.8
	76.0	64.9	60.85
	76.1	64.95	60.9
	76.3	65.2	60.9
	76.2	65.1	61.25
	76.25	65.15	61.2
	76.25	65.15	61.25
	76.1	65.3	60.85
	76.1	65.35	60.95
	76.2	65.5	61.1
Average	76.2	65.2	61.0
F	0.937	0.928	0.924
True Surface Tension	71.4	60.5	56.4

True Surface Tension = Apparent Surface Tension × F

CONCLUSIONS

From the results given, it appears that β-phenylethyl alcohol is not irritating to the rabbit eye when used in 0.3 per cent aqueous solution, that the oral toxicity of the pure chemical to the rat is low, that a 0.3 per cent solution of β-phenylethyl alcohol does not destroy the natural antiseptic action of the lysozyme normally present in the lacrimal fluid and that a 0.3 per cent solution has a slightly lower surface tension than does water and thus would allow better penetration of the ophthalmic solution. In view of the fact that rose water (which contains natural phenylethyl alcohol) has been used for centuries in ophthalmic preparations without harm, the results are not surprising.

REFERENCES

(1) Brewer, J. H., Goldstein, S. W., and McLaughlin, C. B., THIS JOURNAL, **42**, 584(1953).
(2) Lilley, B. D., and Brewer, J. H., *ibid.*, **42**, 6(1953).
(3) Goldstein, S. W., and Ryan, E. P., *Drug Standards*, **20**, 133(1952).
(4) Hjort, A. M., and Eagen, J. T., *J. Pharmacol. Exptl. Therap.*, **14**, 211(1919).
(5) Macht, D. I., *ibid.*, **12**, 263(1918).
(6) Zuidema, H. H., and Waters, G. W., *Ind. Eng. Chem. Anal. Ed.*, **13**, 312(1941).

A Collaborative Study on the Use of Mice in Acute Toxicity Testing*

By O. F. SWOAP†

The 1953 Committee on Physiological Testing of the Scientific Section of the American Pharmaceutical Association evolved a plan of study which was carried out by six different laboratories using the same strain of mice on several diets. The sensitivity of the mice was tested by performing a simple toxicity test at two time intervals on the groups of mice on each diet. This study was undertaken to determine whether or not different laboratories with a given test can obtain results that can be considered statistically equivalent.

THE VALUE of the consideration of the details of various specific testing problems, as usually had been done by the Committee on Physiological Testing of the Scientific Section of the A. PH. A. in previous years, was questioned by the 1951 committee, inasmuch as such problems more logically fell within the scope of the several U. S. P. Revision Committees. The 1951 committee recommended that, in future years, the efforts of the Physiological Testing Committee should be directed toward fundamental problems of physiological testing and that a long range program of work should be planned with the goal of more uniform results of identical tests when conducted in different laboratories.

Such a program is entirely in line with the objectives of the Scientific Section of the A. PH. A. (Chapter I, Article 2 of the By-Laws of the section adopted in 1953). It would encourage high standards of research in the pharmaceutical sciences and would bring individual members and cooperating laboratories closer together for mutual benefit and the exchange of ideas. It was the firm belief of the 1953 committee that it could render a great service to the section and to pharmaceutical science as a whole by continuing this work in the realm of fundamental pharmacology which deals with all pharmacological testing of drugs and chemicals in all areas including toxicity tests, old and new assays, screening tests, et cetera.

The work of the 1948 Physiological Testing Committee (1) followed this principle in a general way. The LD_{50} of several common drugs, determined in eight cooperating laboratories under conditions as nearly identical as possible, showed significant variation between laboratories. It was suggested that these variations in results between laboratories might possibly be due to some slight unrecognized modification in technique for one of the drugs or that differences of the magnitude found might be expected from normal sampling. One recognized variable was that mice of different strains were used by each laboratory.

The 1952 Committee attempted the first phase of the long range program of determining whether or not it is really possible to obtain identical results for the same technique in different laboratories by performing a simple toxicity test on sodium pentobarbital in mice (Webster Swiss)

* Received August 27, 1954 from the Research Laboratories of The Upjohn Company, Kalamazoo, Mich.
Presented to the Scientific Section, A. PH. A., Salt Lake City meeting, August, 1953.
Report of the A. PH. A. Committee on Physiological Testing, 1952–1953.
† Chairman of the A. PH. A. Committee on Physiological Testing, 1952–1953.
As chairman, Mr. Swoap wishes to thank the other members of the Committee: R. P. Ahlquist, M. G. Allmark, K. K. Chen, L. W. Hazleton, H. G. O. Holck, L. C. Miller, J. C. Munch, and L. W. Rowe, and the personnel of the six cooperating laboratories for their splendid cooperation. Acknowledgment is also made to Dr. O. S. Carpenter of the Upjohn Company for technical assistance in interpretation of the data.

from a single colony. The difficulties encountered in completing this work were noted in their report of that year. After completion of the work the question arose as to identity of the mouse strain as the supplier was found to be a broker and not an animal breeder. Also the diets used were not identical. The data presented suggested that identical results could be obtained in different laboratories but the committee concluded that tests of a different design appeared necessary for the final determination of whether or not different laboratories with a given test can obtain results that can be considered statistically equivalent.

The 1953 Committee, after several months of study, met in Chicago, Illinois, during the meetings of the Federation of American Societies for Experimental Biology the week of April 6, 1953. Eight members, or their representatives, were present and the following plan for the year's work was adopted.

PLAN OF STUDY

In keeping with the policy adopted by the committees of 1951 and 1952 of studying fundamental problems of physiological testing, the plan adopted for 1953 consisted of a study of the normal growth curve of groups of mice from a single source on several diets. The sensitivity of the mice was tested by performing a simple toxicity test at two time intervals on the groups of mice on each diet.

Participating Laboratories.—Six laboratories agreed to participate in the year's work. These were as follows:

1. Pharmacology Laboratory, University of Nebraska College of Pharmacy, Lincoln, Nebr. (H. G. O. Holck).[1]
2. Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada (M. G. Allmark).
3. Toxicity Department, Bureau of Fish and Wildlife, U. S. Department of Interior, Denver, Colo. (D. G. Crabtree representing J. C. Munch).
4. Research Laboratories, Parke, Davis and Company, Detroit, Mich. (L. W. Rowe).
5. Research Laboratories, Eli Lilly and Company, Indianapolis, Ind. (R. C. Anderson representing K. K. Chen).
6. Research Laboratories, The Upjohn Company, Kalamazoo, Mich. (O. F. Swoap).

Procedure (General).—The mice used in this test were furnished by Chairman O. F. Swoap, from the Upjohn mouse colony. These were Rockland All Purpose Albino mice from a colony in operation about four years. Two hundred forty weanling mice (120 male and 120 female) weighing from 10–20 Gm. were divided into equal weight groups and shipped to each participating laboratory during the week of April 20th, by the fastest possible route (air or rail express). Immediately upon arrival at each laboratory the mice were separated into two equal groups (sexes kept separate).[2] One group was continued on the Rockland Mouse Diet[3] as used in the breeding colony, and the second group was placed on a second diet of each collaborator's own choice. The mice were given distilled water and fed each diet *ad lib.* for seven days. Forty to fifty representative mice (20–25 male and 20–25 female) from each diet group were then used for the first toxicity test described below. The remaining mice were continued on distilled water and each diet for seven weeks and the toxicity test was then repeated in the same manner. Throughout the seven-week test period individual weight records were kept at one-week intervals. Weighings were made before fresh water and feed were given on the day of weighing.

SPECIFIC DIRECTIONS FOR TOXICITY TESTS

Selection of Mice for Each Test.—Use healthy nonfasted mice weighed to the nearest 0.5 Gm. Arrange the mice so that the average weight in each group will be the same.

Preparation of Test Solutions.—Dissolve 50 mg. of Strychnine Sulfate[4] N. F. (1953 Cooperative A. PH. A. sample) Res. No. 10,277 in distilled water to make 50 cc. (1 mg./cc.) just before the injections are to be made. This solution is then diluted 1–5 with distilled water (10 cc. → 50 cc.) to prepare a 0.2 mg./cc. solution which is then further diluted as necessary for each group to be injected. (These dilutions may be prepared according to Table I.)

TABLE I.—PREPARATION OF TEST SOLUTIONS

Log Dose	Dose, mg./Kg.	Inj., cc./Kg.	Conc., mg./cc.	Use cc. of 0.2 mg./cc.	Prepare cc. Dilution
0.60	4.0	20	0.2	20	20
0.525	3.35	20	0.1675	16.75	20
0.45	2.8	20	0.14	14	20
0.375	2.4	20	0.12	12	20
0.30	2.0	20	0.10	10	20
0.225	1.7	20	0.085	8.5	20
0.15	1.4	20	0.07	7	20
0.075	1.2	20	0.06	6	20
0.00	1.0	20	0.05	5	20

Injection of Mice.—Use a tuberculin syringe of 0.5–1.0 cc. capacity, graduated in 0.01 cc. and fitted with a $^1/_2$–$^5/_8$ inch 24–26 gauge needle. Inject each mouse intraperitoneally. Use 5 male and 5 female mice at each dosage level. Place mice in individual cages for observation.

First Test.—Inject 10 mice (5 male and 5 female) with a dose of 2.0 mg./Kg. Increase or decrease the dosage for each subsequent group of 10 mice at 0.075 log intervals, taken from the above table, until two dosage levels have been used which produce

[1] Dr. Holck wishes to thank the following graduate students for their aid: Messrs. Gale Demaree, Arthur Krieger, Marvin H. Malone, David W. Sjogren, and Miss Helen Chien Fan Su.

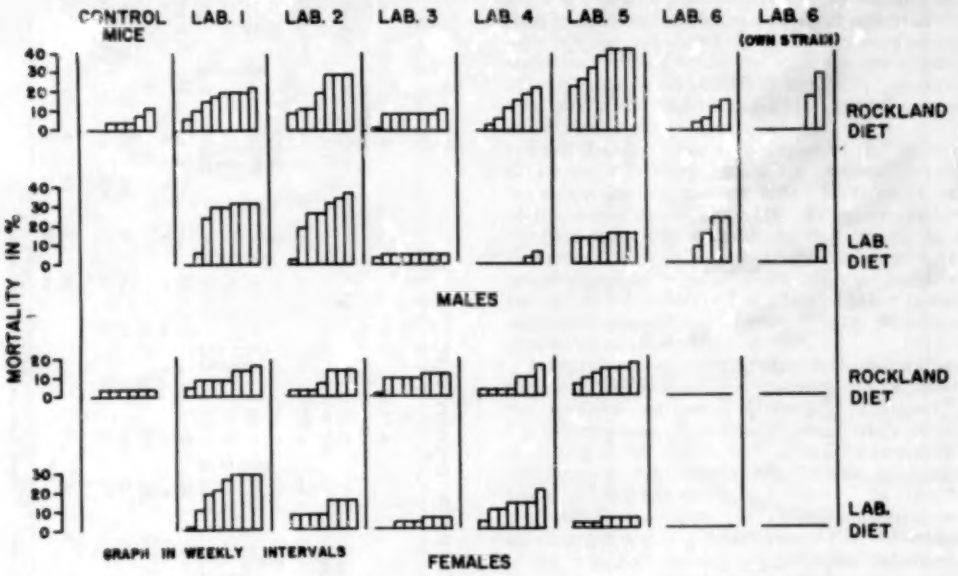
[2] It was recommended that about 4–5 square inches of cage space should be allowed per mouse. (A cage 14 x 24 inches—7 inches high—will hold up to 60–75 mice on shavings.)

[3] One hundred pounds of this diet was sent to each laboratory (a supplement of rolled oats was given the mice during shipment but was not continued after arrival. Raw potatoes were used to furnish water during shipment).

[4] Chosen because of rapidity of action and because the response curve is relatively steep.

CHART I

MORTALITIES OF MICE ON 1953 COOPERATIVE A.Ph.A. TEST DIETS



greater than 50% mortality and two dosage levels have produced less than 50% mortality. (This will use 4 or 5 groups of 10 mice at each level.)

Second Test.—The starting level will be given by Chairman Swoap about a week prior to the second test and will be based on a spot test on mice of corresponding weight. Proceed as in the first test.

Observation of Mice.—Observe and record deaths on each group within a period of two hours. (Deaths from a drug such as strychnine will usually occur within one hour.) Kill the mice which survive this two-hour period.

Other Directions.—Record the maximum and minimum room temperature, and maximum and minimum barometric pressure, of the testing room during the toxicity test. The solutions should be at room temperature when injected.

Record results in black ink or #2 pencil on the ozalid master forms furnished. For convenience a carbon copy may be kept by the testing laboratory and the original should be sent without folding to Chairman Swoap as soon as each test is completed. Ozalid copies of all data will be distributed to each cooperating laboratory and to members of the Committee. (This is the first time that each cooperative laboratory has received copies of work sheets from other laboratories.)

HANDLING AND SHIPMENT OF MICE

About 1500 mice were weaned on Monday, April 20th, sexed, and separated into weight groups from 10–20 Gm. at 2-Gm. intervals. Tuesday, April 21st, the mice were divided without reweighing into six groups of 120 males and six groups of 120 females. Twenty-five males and 25 females were also kept as normal controls. That evening the mice were boxed for shipment in ventilated waxed cardboard boxes (14″ x 18″ × 7″ high) containing cedar shavings (120 mice per box). Four hundred grams of diet was added and also about four hundred grams of potatoes to furnish water during shipment. The boxes were assigned to each laboratory at random and were held in the animal room until Wednesday morning. Shipments were made by air to Ottawa, Lincoln, Denver, and Indianapolis, and by rail to Detroit and were completed by 10:00 p. m. To simulate treatment of the other mice, those for use in the Upjohn laboratory were taken to the toxicity animal room and kept boxed until Thursday morning, April 23rd, and then placed on the test diets.

RESULTS

Mortality of Mice on Test Diets.—Losses during shipment and during the first week of feeding ran from 9–24 mice per laboratory or 4–10% of the total mice. The Upjohn group of mice showed the greatest loss during this period possibly because they were not moved as much during the simulated shipment period allowing more chance for overcrowding. The mortalities of the mice on the test diets during the seven-week test period are shown as bar-graphs in Chart I. They all appear to be within normal limits.

Growth Curves on Test Diets.—The growth of all mice on the common diet and on the individual laboratory diets appear to be similar. These are graphically represented in Chart II. This test shows that mice from a common source can be maintained satisfactorily in different laboratories on any one of the several nutritionally adequate diets tested. Minor differences may be due to small differences in the growth factors of the diet, care,

TABLE II.—FIRST RUN—1953 COOPERATIVE A. PH. A. TOXICITY TEST

Lab. No.	1				2				3				4				5				6							
Date	4-30-53				4-30-53				4-30-53				5-2-53				4-29-53				4-30-53							
Room Temp.	78–77° F.				80° F.				79–81° F.				76–79° F.				77° F.				80–82° F.							
Barometric Pressure	728.9–727.7 mm.				741.7 mm.				733.6 mm.				721.1–722.4 mm.				614.7 mm.				746.8–756.9 mm.							
Mice Used	Test				Test				Test				Test				Test				Test				Own Strain			
Diet	Rockland		Nutrition Breeders		Rockland		Breeders		Rockland		Purina Lab. Chow		Rockland		Purina Lab. Chow		Rockland		Purina Fox Chow		Rockland		Master Fox Breeders		Rockland		Master Fox Breeders	
Sex→	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
2.8 mg./Kg.	100[a]	80[a]	100[a]	100[a]	a	a	a	a	a	a	a	a	a	a	a	a	a	a	80[a]	100[a]	a	a	a	a	a	a	a	a
2.4 mg./Kg.	100	40	100	40	100	80	80	40	80	100	100	100	100	100	100	100	100	100	100	100	..	..	..	..	..	..	..	..
2.0 mg./Kg.	20	0	40	0	80	80	40	20	80	40	60[b]	30[b]	80	80	40	20	100	100	20	0	80	80	80	100	100	80	80	100
1.7 mg./Kg.	0	0	0	0	40	60	40	20	40	20	0	0	60	40	40	0	20	20	0	0	80	40	40	60	80	60	100	60
1.4 mg./Kg.	..	..	..	..	40	20	0	0	0	20	..	..	20	0	40	20	0	0	..	..	20	0	0	20	20	0	80	20
1.2 mg./Kg.	..	..	..	..	..	..	..	..	..	..	..	..	0	20	0	20	..	..	..	..	0	20	0	0	20	0	20	40

[a] Percentage mortality based on 5 mice. [b] Ten mice per group at this level.

TABLE III.—SECOND RUN—1953 COOPERATIVE A. PH. A. TOXICITY TEST

Lab. No.	1				2				3				4				5				6							
Date	6-18-53				6-18-53				6-18-53				6-20-53				6-18-53				6-18-53							
Room Temp.	76–78° F.				75° F.				78–79° F.				76–79° F.				78° F.				78–80° F.							
Barometric Pressure	760.1–756.7 mm.				748.0 mm.				745.4 mm.				720.1–722.1 mm.				623.6 mm.				751.8 mm.							
Mice Used	Test				Test				Test				Test				Test				Test				Own Strain			
Diet	Rockland		Nutrition Breeders		Rockland		Breeders		Rockland		Purina Lab. Chow		Rockland		Purina Lab. Chow		Rockland		Purina Fox Chow		Rockland		Master Fox Breeders		Rockland		Master Fox Breeders	
Sex→	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
2.8 mg./Kg.	a	a	a	a	a	a	a	a	100[a]	100[a]	80[a]	100[a]	a	a	a	a	a	a	100[a]	100[a]	a	a	a	a	a	a	a	a
2.4 mg./Kg.	100	100	100	100	..	..	..	..	40[b]	50[b]	60	20	..	..	100	80	100	100	100	100	..	..	..	..	..	..	..	..
2.0 mg./Kg.	100	60	100	80	80	100	80	50[b]	0	20	20	40	80	60	40	60	100	100	40	40	100	60	100	80	80	80	100	100
1.7 mg./Kg.	60	0	80	20	100	60	60	60	..	..	0	0	100	60	20	0	40	0	40	0	80	60	100	60	40	60	100	60
1.5 mg./Kg.[c]	..	..	..	..	80	20	60	0	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
1.4 mg./Kg.	60	0	0	20	20	40	0	0	..	..	..	..	40	20	20	0	0[d]	0	..	..	40	40	80	0	0	0	80	20
1.2 mg./Kg.	0[d]	0	0[a]	0	20	0	20	0	..	..	..	..	0	0	..	..	..	..	..	..	20	20	40	0	..	..	20	0

[a] Percentage mortality based on 5 mice. [b] Ten mice per group at this level. [c] Extra level used by Lab. #2 only. [d] Three mice per group at this level.

TABLE IV.—ANALYSIS OF 1953 COOPERATIVE A. PH. A. TOXICITY TESTS—MILLER-TAINTER METHOD[a]

LD_{50} ± Std. Error, mg./Kg.

Lab. No.	Mice	Males: Run I LD_{50} ± S. E.	Males: Run I Rank	Males: Run II LD_{50} ± S. E.	Males: Run II Rank	Females: Run I LD_{50} ± S. E.	Females: Run I Rank	Females: Run II LD_{50} ± S. E.	Females: Run II Rank	Avg. S. E.	Differences Within Labs.—M-F: Run I-II, Males D	Males D/S. E.	Females D	Females D/S. E.	Run I D	Run I D/S. E.	Run II D	Run II D/S. E.
1	Test	2.08 ± 0.06	1	1.54 ± 0.06	5	2.48 ± 0.08	1	1.85 ± 0.08	5	0.07	0.54	8	0.63	9	−0.40	5	−0.31	4
2	Test	1.78 ± 0.08	4	1.70 ± 0.08	4	2.02 ± 0.06	2	1.90 ± 0.06	3	0.07	0.08	1	0.12	1	−0.24	3	−0.20	3
3	Test	1.90 ± 0.08	3	2.32 ± 0.08	1	1.82 ± 0.06	5	2.22 ± 0.08	1	0.08	−0.42	5	−0.40	5	0.08	1	0.10	1
4	Test	1.74 ± 0.10	5	1.74 ± 0.10	3	1.83 ± 0.07	4	1.90 ± 0.07	3	0.08	0.00	0	−0.07	1	−0.09	1	−0.16	2
5	Test	2.05 ± 0.08	2	1.80 ± 0.08	2	1.96 ± 0.06	3	1.96 ± 0.06	2	0.07	0.25	3	0.07	0	0.09	1	0.16	2
6	Test	1.72 ± 0.06	6	1.36 ± 0.06	6	1.65 ± 0.08	6	1.68 ± 0.08	6	0.07	0.36	5	−0.0	0.5	0.07	1	0.32	4
6	Own strain	1.47 ± 0.08		1.47 ± 0.08		1.62 ± 0.06		1.65 ± 0.06		0.07	0.00	0	−0.03	0.5	−0.15	2	−0.18	2
Ranges between labs.	Test																	
	Expt.	0.36		0.96		0.53		0.54										

[a] *Proc. Soc. Exptl. Biol. Med.*, **57**, 261 (1944).

housing, etc., but this is not reflected in the death rate (Chart I) or the toxicity tests reported below.

Toxicity Tests.—*Individual Tests at One- and Eight-Week Periods.*—These tests were set up to determine the effect of diet, sex, age, and weight of the mice on the calculated LD_{50}. They were necessarily limited to a relatively small number of mice at each dosage level because of the limitation of the total number of mice available and in order to keep the work as simple as possible.

Both tests were run with a starting dosage level of 2.0 mg. strychnine sulfate/Kg. Mortalities obtained on the first run are shown in Table II and on the second run in Table III. An examination of the data in these tables indicates that differences in barometric pressure and small differences in room temperatures have no apparent effect on the LD_{50} for strychnine sulfate. The effect of other factors mentioned above was examined after statistical evaluation of the results.

Interpretation of Results.—The LD_{50} values and their standard errors were calculated using the shortcut graphical method of Miller and Tainter (2). No difference was found between the diets on the same day so the results were combined to test sex differences giving a minimum of 10 mice per dosage level. Results are shown in Table IV. In examining Table IV we note that: (*a*) The standard errors are all about .07 mg./Kg. which means that differences of 0.2 mg./Kg. upward are probably significant; (*b*) Laboratory 1 observed a significant difference between sexes in the first run but probably not in the second. None of the sex differences observed in the other laboratories can be said to be really different and very probably the sex difference observed from the combined results of all laboratories is not statistically significant; (*c*) Laboratories 1 and 3 observed a significant difference between Runs I and II but in opposite directions; Laboratory 1 obtained a lower LD_{50} in Run II while Laboratory 3 obtained a higher value. Other laboratories showed no real differences; (*d*) In any one run the variation among laboratories is significant with Laboratory 6 always having the lowest LD_{50}. Laboratories 1 and 3 were generally the highest; (*e*) Laboratory 6 found no real difference between the mice supplied and its own strain; (*f*) There is a strong suggestion that the slopes of the dosage mortality curves differ from laboratory to laboratory.

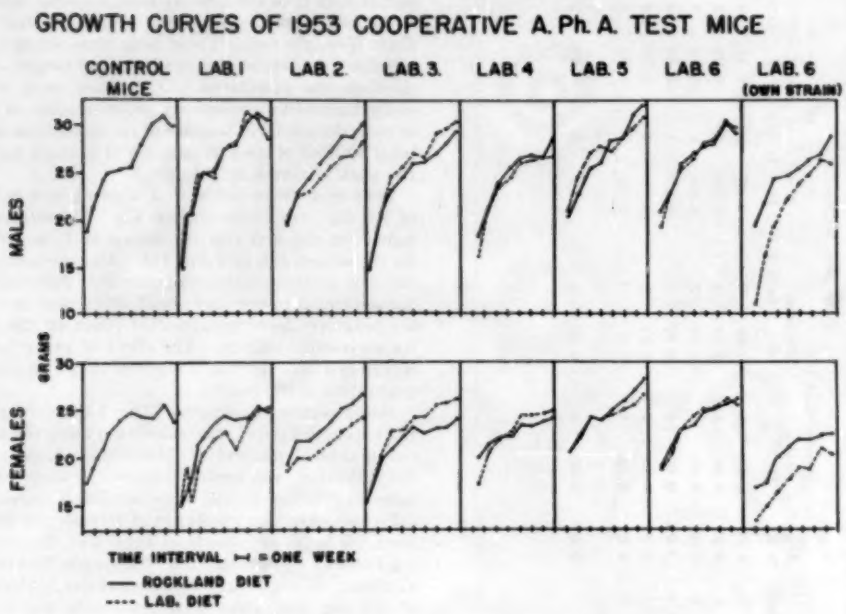
The LD_{50} values were also calculated by the Behhrens (3), Karber (4), and Berkson (5) methods. Individual results by these methods are omitted for simplicity of the report but they were found to agree, in general, with those obtained by the Miller-Tainter (2) method recorded in Table IV, except that a possible real difference between diets was suggested in the results from some laboratories.

CONCLUSIONS

1. Mice from a common source can be maintained satisfactorily in different laboratories on any one of the several nutritionally adequate diets tested.

2. Small differences in room temperatures have no apparent effect on the observed differences in LD_{50} values.

CHART II



3. Differences in altitude or barometric pressure are apparently not responsible for the observed differences in LD_{50} values.

4. Differences in the strains of mice used are apparently not responsible for the observed differences in LD_{50} values. This is worth rechecking later but should be postponed until it is demonstrated that different laboratories can obtain results that are statistically equivalent on mice from a single source.

5. Minor variations in technique should be examined, both within and between laboratories, in an attempt to account for the observed differences in results in the above test.

6. A comparative study of methods of estimation of LD_{50} values is suggested in order better to ascertain which methods are to be preferred for practical work.

REFERENCES

(1) Allmark, M. G., THIS JOURNAL, **40**, 27(1951).
(2) Miller, L. C., and Tainter, M. L., *Proc. Soc. Exptl. Biol. Med.*, **57**, 261(1944).
(3) Burn, J. H., "Biological Standardization," 2nd ed., Oxford University Press 1950, p. 11.
(4) Finney, D. J., "Statistical Method in Biological Assay," Hafner, New York, 1952, p. 524.
(5) Berkson, J., *J. Am. Stat. Assoc.*, **48**, 565(1953).

A Note on Tablet Disintegration with Starch*

By LEMUEL C. CURLIN†

STARCH has been used as a disintegrant in tablets for decades. Its prompt action is well shown in the common aspirin tablet, which will break apart in seconds when dropped into water. This disintegrating action was thought to be due to starch granules swelling in water and breaking open the tablet. Other agents, which swell in water, as bentonite, gums, etc. have been used as disintegrants with poor results.

We recently tried disintegrating aspirin tablets in hot water. They did not break up but remained firm for a long period of time. Hot water should swell the starch granules quickly and break the tablets faster. We microscopically examined a thin slurry formed by dropping an aspirin tablet into a small amount of cold water. Although the tablet disintegrated in 15 seconds, the starch granules were not swollen.

We applied a drop of dye solution to an aspirin tablet. On cutting the tablet in half, we found the color had penetrated the tablet as though it were a blotter.

We suggest that the disintegrating action of starch in tablets is due to capillary action rather than swelling. Possibly the spherical shape of the starch increases the porosity of the tablet.

* Received December 4, 1953 from L. Perrigo Company, Allegan Mich.
† Chief Chemist, L. Perrigo Company.

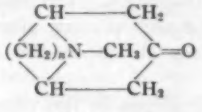
A Synthesis of Pseudopelletierine*

By BLAKE F. PUTNEY† and TAITO O. SOINE

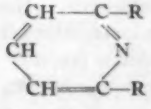
A new synthesis of pseudopelletierine is presented in which the diethyl ester of lobelinic acid was subjected to a Dieckmann type condensation to yield carbethoxypseudopelletierine which on hydrolysis and decarboxylation gave pseudopelletierine. A total synthesis of lobelinic acid was also accomplished by reduction of pyridyl-2,6-diacetic acid dianilide, followed by hydrolysis to give norlobelinic acid which was methylated by treatment with formaldehyde and formic acid to give lobelinic acid. Several new compounds prepared include pyridyl-2,6-diacetic acid diethyl ester hydrochloride; norlobelinic acid, its anilide and its dimethyl and diethyl esters as hydrochloride salts; and dimethyl and diethyl esters of lobelinic acid as hydrochloride salts.

PSEUDOPELLETIERINE (I) was first prepared by Menzies and Robinson (1) by the interaction of glutaraldehyde, calcium acetonedicarboxylate, and methylamine at a controlled *p*H. This synthesis was carried out under physiological conditions in an effort to simulate the biogenesis of the alkaloid and was essentially the same procedure used by Robinson (2) earlier to obtain tropinone (II). The yields in this synthesis were very poor and the method was further handicapped by the use of difficult-to-obtain glutaraldehyde as a starting material. The same reaction under varying conditions has been reported by Schöpf and Lehman (3), Ziegler and Wilms (4) and Cope, *et al.* (5). The latter group reported yields as high as 42 to 55 per cent when working with molar quantities. From a consideration of the above reports it was thought worth while to investigate the synthesis of pseudopelletierine with the objective of devising, if possible, a more convenient procedure obviating the use of glutaraldehyde.

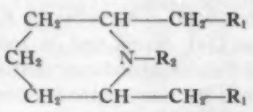
The diethyl ester of lobelinic acid (*N*-methylpiperidyl-2,6-diacetic acid) (III) was considered a likely starting material for the synthesis. An inner Dieckmann type condensation would serve to effect ring closure in a manner similar to that used by Willstätter (6) for the preparation of tropinone from the diethyl ester of N-methylpyrrolidine-2,5-diacetic acid.



I. $n = 3$
II. $n = 2$

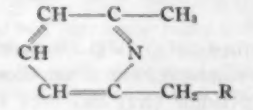


IV. R = CH_3
V. R = COOH



VI. R = $COCHN_2$
VII. R = CH_2COOH
VIII. R = $CH_2COC_6H_5$
XI. R = $CH_2C(NOH)C_6H_5$
XII. R = $CH{=}CHC_6H_5$
XIII. R = $CHBrCHBrC_6H_5$
XIV. R = $C{\equiv}CC_6H_5$
XV. R = $CH_2CONHC_6H_5$

III. R_1 = $COOC_2H_5$, R_2 = CH_3
XVI. R_1 = COOH, R_2 = H
XVII. R_1 = COOH, R_2 = CH_3



IX. R = $COOC_2H_5$
X. R = COC_6H_5

Previous work in this laboratory (7, 8) had dealt with the oxidation of 2,6-lutidine (IV) to dipicolinic acid[1] (V). This acid, as the dichloride, was envisioned as a good stepping-off point for the synthesis of lobelinic acid which previously had been prepared by Wieland and Dragendorff (9) in poor yield by a Beckmann rearrangement of the dioxime of lobelanine. The Arndt-Eistert reaction (10), applied to dipicolinic acid, appeared to be a likely method for obtaining the desired diethyl lobelinate. Unfortunately, suitable conditions for effecting the Wolff rearrangement

* Received August 30, 1954, from the Department of Pharmaceutical Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, Minn.

† Samuel W. Melendy Fellow, 1950–1952. Present address: College of Pharmacy, Rutgers University, Newark, N. J.

[1] Pyridine-2,6-dicarboxylic acid.

of 2,6-bis-diazoacetylpyridine (VI) could not be found. The unusual stability of the diazo compound perhaps may be accounted for by its resonance forms. The resistance of the diazoketone to rearrangement led to the elimination of this method of synthesis for the present problem.

It is well known (12) that the ethyl ester of pyridyl-2-acetic acid is obtainable by reaction of 2-picoline with phenyl lithium followed by direct carbonation of the picolyl-lithium complex and subsequent esterification of the formed acid. The application of similar reactions to the homologous 2,6-lutidine appeared as an attractive possibility for the synthesis of the desired compound. The literature concerning the use of the lithium complex of 2,6-lutidine is, however, somewhat contradictory. Bergman and Pinchas (13) reported the synthesis of some 2,6-bilaterally substituted pyridines by reacting the lithium complex of 2,6-lutidine with aromatic aldehydes. On the other hand the studies of Kloppenburg and Wibaut (14), Sixma and de Jong (15) and Barnes and Fales (16) indicate that monolateral substitution is the more common occurrence. In the present paper, an exploration into the reaction of phenyl lithium with 2,6-lutidine followed by carbonation of the lithium complex left little doubt that the desired bilaterally substituted compound is not obtained in significant amounts. These results led to the abandonment of this synthetic route.

The synthesis of pyridyl-2,6-diacetic acid by Beckmann rearrangement of the dioxime of 2,6-diphenacylpyridine (XI) had been reported by Oparina (11). This method was based on the previous preparation of 2,6-diphenacylpyridine by Scheuing and Winterhalder (17). It was decided to adapt their previous method of synthesis to the present problem.

The first step in the series of reactions involves the preparation of 2,6-distyrylpyridine (18, 19, 20) (XII) by condensing benzaldehyde with 2,6-lutidine. The bromination of 2,6-distyrylpyridine had been reported by Schuster (21) and Shaw (22) and was found to proceed as reported. Dehydrobromination of the resulting 2,6-distyrylpyridine tetrabromide (XIII) to 2,6-di-(β-phenylethinyl)pyridine (XIV) and hydration of the latter product to 2,6-diphenacylpyridine by the procedure of Scheuing and Winterhalder (17) were of necessity altered to improve yields. The dioxime of 2,6-diphenacylpyridine was prepared and rearranged as previously reported by Oparina (11). The product, pyridyl-2,6-diacetanilide (XV), was found to agree closely with the product reported by the above author. On hydrolysis, pyridyl-2,6-diacetic acid was obtained but had a melting point somewhat higher than that recorded in the literature. However, the melting point and analysis of the methyl ester agreed with the expected values. The diethyl ester, previously unreported, was prepared as the hydrochloride.

Pyridyl-2,6-diacetic acid is very susceptible to decarboxylation and, for this reason, the pyridine-ring was reduced before hydrolyzing the dianilide. The hydrogenation, utilizing platinum oxide, was carried out using glacial acetic acid as solvent in one instance and absolute methanol containing an equivalent of hydrogen chloride in the other. Products having decidedly different melting points were obtained from the hydrogenations carried out in the different solvents. However, both reduced products gave piperidyl-2,6-diacetic acid (norlobelinic acid) (XVI) on hydrolysis. The dimethyl and diethyl esters of this acid were prepared as the hydrochlorides. N-methylation of nor-lobelinic acid was effected by the use of formaldehyde and formic acid as described by Clarke (23). The product, N-methyl-piperidyl-2,6-diacetic acid (lobelinic acid) (XVII) was obtained in good yield and corresponded in melting point and analysis to the expected values. The dimethyl and diethyl esters of this acid were prepared as the hydrochlorides.

The Dieckmann condensation of diethyl lobelinate was carried out in a manner similar to that described by Fuson (24) for preparation of piperidone compounds. The product obtained after decarboxylation of the intermediate product was slightly yellow in color and had a melting point of 44–45°. Cope (5) had observed that, on exposure to atmospheric moisture, pseudopelletierine formed a hemihydrate, m. p. 48–49°. He also obtained the anhydrous form, m. p. 64–65° by repeated sublimation. The pseudopelletierine prepared in the present work, after sublimation, was identical in all respects to the authentic alkaloid.[1]

The work described in this paper shows that pseudopelletierine can be synthesized from diethyl lobelinate in yields comparable to those utilizing glutaraldehyde. However, until a shorter and more convenient synthesis of diethyl lobelinate is devised it probably has little advantage over the previous methods.

EXPERIMENTAL

2,6-Bis-Diazoacetylpyridine.—A solution of diazomethane (prepared from 50 Gm. of N-nitrosomethyl-

[1] The authors wish to acknowledge with gratitude the kind help of Dr. A. C. Cope of the Massachusetts Institute of Technology, Cambridge, Mass., in supplying us with a generous sample of authentic pseudopelletierine.

urea) in 500 cc. of ether was placed in a 2-L., 3-necked flask equipped with a mercury-sealed stirrer, reflux condenser, and dropping funnel. Dipicolinic acid dichloride, previously prepared from 10 Gm. of hydrated dipicolinic acid (8), was dissolved in 200 cc. of absolute ether and added slowly to the solution of diazomethane through the dropping funnel; stirring and cooling being maintained during the addition. About 6 Gm. of a bright yellow crystalline compound precipitated from the ethereal solution and was removed by filtration. Concentration of the mother liquors yielded an additional 2 Gm. of diazoketone bringing the total yield to 8 Gm. (68%). The product could be crystallized from absolute ethanol but was found to be somewhat unstable on exposure to light and air although it could be preserved unchanged for short periods of time in a refrigerator.

Anal.—Calcd. for $C_9H_6N_4O_2$: N, 32.6. Found: N, 30.6.

Attempted Rearrangement of 2,6-Bis-Diazoacetylpyridine.—The usual methods (10) for effecting the Wolff rearrangement to obtain either the desired acid or the corresponding ester were employed with no success. Extended reflux periods and higher boiling solvents were likewise without value and unchanged diazoketone could be isolated from the reaction mixture in each case.

Reaction of 2,6-Lutidine with Phenyl Lithium.—The reaction of 2,6-lutidine (0.5 mole) with phenyl lithium (1 mole) was carried out in the manner described for the synthesis of the ethyl ester of pyridyl-2-acetic acid (12). Fractionation of the product yielded a forerun of unreacted 2,6-lutidine followed by two distinct products of the reaction. The first fraction, distilling at 110–120°/10 mm., was concluded to be 6-(2-picolyl) acetic acid ethyl ester on the basis of the analytical results. The hydrochloride salt of the ester, m. p. 114–116°, was prepared according to the usual methods and used for the analyses.

Anal.—Calcd. for $C_{10}H_{13}O_2N \cdot HCl$: C, 55.7; H, 6.5; N, 6.5; Cl, 16.2. Found: C, 55.7; H, 6.5; N, 6.7; Cl, 16.4. Calcd. for $C_{10}H_{13}O_2N \cdot HCl$: Saponification equivalent, 108. Found: 101.

The second fraction, distilling at 170–180°/2 mm., solidified on cooling and was crystallized from dilute ethanol, m. p. 77°. A 2,4-dinitrophenylhydrazone, m. p. 218–222°, was prepared in the usual manner. Kloppenburg and Wibaut (14) reported a compound they assumed to be 6-(2-picolyl)benzoyl methane and it is believed to be the same as the compound obtained here. The picrate, m. p. 182–183°, corresponded very closely to their picrate also. The previously unreported hydrochloride, m. p. 193–195°, was prepared.

Anal.—Calcd. for $C_{14}H_{13}NO$: C, 79.6; H, 6.2; N, 6.6. Found: C, 79.3; H, 6.3; N, 6.7. Calcd. for $C_{14}H_{13}NO \cdot HCl$: C, 67.9; H, 5.7; N, 5.7; Cl, 14.3. Found: C, 67.9; H, 5.8; N, 5.8; Cl, 14.2.

2,6-Distyrylpyridine.—The condensation of 2,6-lutidine with benzaldehyde was effected in 45% yield in a manner similar to methods reported in the literature (18, 19, 20). The product obtained here, after crystallization from benzene and ethanol, had a melting point of 164–165°. The literature (20) records m. p. 167.5° for this compound.

2,6-Distyrylpyridine Tetrabromide.—Bromination of 2,6-distyrylpyridine was carried out according to the method of Shaw (22). The product was crystallized from carbon tetrachloride in a yield of 90%, m. p. 180°. The literature gives melting points of 179° (22) and 183° (21) for this compound.

2,6-Di-(β-phenylethinyl)pyridine.—Dehydrobromination of distyrylpyridine tetrabromide was carried out according to Scheuing and Winterhalder (17) except that longer reflux periods were found to be necessary. The product was obtained in 62% yield, m. p. 138–139°. The literature (17) reports m. p. 137–138°.

2,6-Diphenacylpyridine.—Hydration of 2,6-di-(β-phenylethinyl)pyridine was carried out by the method of Scheuing and Winterhalder (17). Crystallization from ethanol yielded 2,6-diphenacylpyridine sulfate in 70% yield, m.p. 195°. The above authors reported 197° as the melting point of this compound.

The free base was obtained from the above salt in 90% yield, m.p. 90°. The authors reported 92° as the melting point of 2,6-diphenacylpyridine.

2,6-Diphenacylpyridine Dioxime.—The oxime was prepared in the usual manner in a yield of 89%, m. p. 174–5°. Oparina (11) reports a melting point of 176° for this compound.

Pyridyl-2,6-diacetanilide.—The Beckmann rearrangement of 2,6-diphenacylpyridine dioxime was carried out according to the method of Oparina (11). The product was first washed with chloroform and then crystallized from ethanol yielding pyridyl-2,6-diacetanilide in 40% yield, m. p. 203–4°. The reported melting point is 198°.

Norlobelinic Acid Dianilide (Piperidine-2,6-diacetanilide).—Pyridine-2,6-diacetanilide (15 Gm.) was suspended in 200 cc. of glacial acetic acid, 0.5 Gm. of platinum oxide catalyst was added and the mixture placed in a Parr low pressure hydrogenator at an initial pressure of 45 lb. of hydrogen. In about six to eight hours all of the anilide had dissolved and the equivalent of approximately 3 moles of hydrogen had been absorbed. The catalyst was removed by filtration and the acetic acid was removed from the filtrate by distillation under reduced pressure. The syrupy residue was taken up in water, neutralized with sodium carbonate and yielded about 8 Gm. of a white crystalline substance. Crystallization from ethanol and water gave a product, m. p. 198°.

Anal.—Calcd. for $C_{21}H_{23}N_3O_2$: C, 71.8; H, 7.2. Found: C, 72.3; H, 7.8.

Norlobelinic Acid (Piperidyl-2,6-diacetic Acid).—Ten grams of the anilide from the previous experiment was boiled under reflux in 200 cc. of concentrated hydrochloric acid for three hours. The mixture was evaporated to dryness on a steam bath and the residue taken up in 100 cc. of water and evaporated to dryness again. This was repeated once more and finally the residue was taken up in 100 cc. of water and neutralized while hot with silver carbonate. The precipitated silver chloride was removed by filtration and the filtrate saturated with hydrogen sulfide. After removal of the precipitated silver sulfide by filtration, the filtrate was concentrated under vacuum until crystallization was noted. The concentrated solution was placed in a 50-cc. beaker and the flask rinsed with ethanol to remove remaining acid. The combined solution and rinsings were cooled and the precipitated acid

removed by filtration and the filtrate was then concentrated to obtain any acid remaining in the mother liquor. Approximately 3.5 Gm. (60%) of nor-lobelinic acid, m. p. 230–231° (efferv. at 160–170° with resolidification) was obtained. The product analyzed for one mole of water of hydration. After drying at 110° for twelve hours in an evacuated Abderhalden drying pistol the product melted at 230–231° without preliminary effervescence and analyzed for the expected formula without water of hydration.

Anal.—Calcd. for $C_9H_{13}NO_4 \cdot H_2O$: C, 49.3; H, 7.8; N, 6.4. Found: C, 49.5; H, 7.9; N, 6.5. Calcd. for $C_9H_{15}NO_4$: C, 53.7; H, 7.5; N, 7.0. Found: C, 53.1; H, 7.6; N, 6.8.

Pyridyl-2,6-diacetic Acid.—This acid was prepared from pyridyl-2,6-diacetanilide in the same manner used to prepare nor-lobelinic acid. The product obtained in this work had a melting point of 152° (decomp.) while that reported in the literature (11) was 142°. The reason for the discrepancy in melting points is not known.

Pyridyl-2,6-diacetic Acid Dimethyl Ester Hydrochloride.—This ester was prepared from the above acid in two ways.—*Diazomethane Method.*—One gram of pyridyl-2,6-diacetic acid was added to 50 cc. of cold ether containing diazomethane (prepared from 5 Gm. of N-nitrosomethylurea) and allowed to dissolve. When the acid was completely dissolved the ethereal solution was concentrated almost to dryness at which point most of the dimethyl ester crystallized out. The crude product weighed 1 Gm. (90%). It was crystallized from petroleum ether to obtain an analytical sample, m. p. 62° which was in agreement with the melting point of 62° reported by Oparina (11). *Alcoholysis of the Anilide.*—Pyridyl-2,6-diacetanilide (10 Gm.) was placed in 300 cc. of absolute methanol and heated for three hours under reflux while passing in dry hydrogen chloride gas. The system was protected from moisture with a calcium chloride drying tube. After the reflux period the solvent was removed under reduced pressure, the residue taken up in 150 cc. of water and then alkalized with sodium carbonate. The water was removed under reduced pressure on a steam bath, the residue taken up in 150 cc. of water again and finally concentrated to dryness. The residue was taken up in 5% (w/v) hydrochloric acid solution, alkalized with sodium carbonate and extracted with ether. The ethereal extract was dried over anhydrous sodium sulfate and the ether removed to yield a slightly colored crystalline residue. Upon crystallization from petroleum ether 3.3 Gm. (50%) of pyridyl-2,6-diacetic acid dimethyl ester, m. p. 62° was obtained. It was identical to the product obtained by the diazomethane method as determined by a mixed melting point.

Anal.—Calcd. for $C_{11}H_{13}NO_4$: C, 59.2; H, 5.9. Found: C, 59.6; H, 6.1.

Pyridyl-2,6-diacetic Acid Diethyl Ester Hydrochloride.—This ester was prepared by the alcoholysis of the corresponding anilide in ethanol saturated with hydrogen chloride gas. The procedure is essentially the same as that described above for the dimethyl ester. The product, isolated as its hydrochloride salt in 52% yield, has m. p. 133–134°.

Anal.—Calcd. for $C_{13}H_{17}NO_4 \cdot HCl$: C, 54.3; H, 6.0. Found: C, 54.2; H, 6.2.

Dimethyl Nor-lobelinate Hydrochloride.—Pyridyl-2,6-diacetic acid dimethyl ester hydrochloride (2 Gm.) was dissolved in 75 cc. of absolute ethanol and an equivalent amount of alcoholic hydrogen chloride was added. The mixture was reduced in a Parr low pressure hydrogenator at an initial pressure of 45 lb. using 0.2 Gm. of platinum oxide as catalyst. When absorption of hydrogen had ceased, the catalyst was filtered from the solution, the solvent removed under reduced pressure and the residue crystallized by rubbing with absolute ether. After cooling for several hours, the product was removed by filtration and recrystallized from ethyl acetate. The yield was quantitative. The melting point of the product after repeated crystallization from ethyl acetate was 168°.

This ester was also prepared by reaction of diazomethane with nor-lobelinic acid in the same manner as the conversion of pyridyl-2,6-diacetic acid to its methyl ester previously described. On conversion to the hydrochloride salt and crystallization from ethyl acetate the product, m. p. 168°, was obtained. It was identical to the product prepared by reduction of the corresponding pyridine compound. The analytical results, however, of both compounds were not in agreement with the calculated values. It is believed that the compound has picked up ethyl acetate of crystallization during the crystallization procedure but this could not be removed by prolonged heating at 110° in an evacuated Abderhalden pistol. The analytical values agree very well for one mole of ethyl acetate of crystallization in this compound.

Anal.—Calcd. for $C_{11}H_{19}NO_4 \cdot HCl \cdot C_4H_8O_2$: C, 50.9; H, 7.9. Found: C, 51.1; H, 7.9.

Diethyl Norlobelinate Hydrochloride.—Pyridyl-2,6-diacetic acid diethyl ester hydrochloride was dissolved in absolute ethanol and reduced in a Parr low pressure hydrogenator using platinum oxide as a catalyst in the same manner as employed for the corresponding methyl ester. The product was crystallized from ethyl acetate, m. p. 185°.

Anal.—Calcd. for $C_{13}H_{23}NO_4$: C, 53.1; H, 8.2. Found: C, 52.7; H, 8.5.

Lobelinic Acid (N-methyl-piperidyl-2,6-diacetic Acid).—Nor-lobelinic acid (10 Gm.) was placed in a 25-cc. flask and 4.8 cc. of a 40% solution of formaldehyde and 7.6 cc. of a 90% solution of formic acid were added. The mixture was heated under reflux until effervescence had ceased (12 hours or more). The mixture was filtered and evaporated to dryness on a steam bath. The residue was taken up twice in 50 cc. of water and each time evaporated to dryness. When the product started to crystallize on the final evaporation, 100 cc. of ethanol was added and the mixture allowed to cool. The precipitated lobelinic acid, about 7 Gm. (63%), was collected on a filter and recrystallized from water and ethanol to give a white product, m. p. 223.5°. The literature (9) reports 225–228° as the melting point for this compound from natural sources.

Anal.—Calcd. for $C_{10}H_{17}NO_4$: C, 55.8; H, 8.0. Found: C, 55.7; H, 8.0.

Dimethyl Lobelinate Hydrochloride.—Dimethyl nor-lobelinate hydrochloride (2 Gm.) was dissolved in 100 cc. of absolute methanol and an equivalent quantity of 40% formaldehyde solution was added. The mixture was hydrogenated at 70° in a Parr apparatus using platinum oxide (0.2 Gm.) as a

catalyst. The catalyst was removed by filtration and the solvent by distillation under reduced pressure. The residue was dissolved in ethyl acetate with the aid of heat and on cooling a product was obtained which undoubtedly was unreacted starting material as judged by its melting point and mixed melting point. Addition of absolute ether to the mother liquor yielded a product which, after repeated crystallization from ethyl acetate and ether, had a melting point of 131–132°.

Anal.—Calcd. for $C_{12}H_{21}NO_4 \cdot HCl$: C, 51.5; H, 7.9. Found: C, 51.0, H, 7.9.

Diethyl Lobelinate.—Lobelinic acid (2 Gm.) was suspended in absolute ethanol (200 cc.) and placed in a flask equipped with a reflux condenser surmounted by a drying tube and a side arm admitting a gas inlet tube beneath the surface of the liquid. While heating, dry hydrogen chloride gas was passed through the solution for a total of four hours. The solvent was removed under reduced pressure and the residue was alkalized with sodium carbonate solution. The alkaline solution was extracted with ether and dried over anhydrous sodium sulfate and Drierite® for six to eight hours. The ether was removed and the residue distilled at 149–150°/4 mm. (175–177°/12 mm.). The hydrochloride salt was prepared by the addition of ethereal hydrogen chloride to the dried ethereal solution of the ester and crystallized from ethyl acetate and ether, m. p. 124°.

Anal.—Calcd. for $C_{14}H_{25}NO_4 \cdot HCl$: C, 54.6; H, 8.5. Found: C, 54.6; H, 8.4.

The ester was also prepared by addition of an excess of an ethereal solution of diazoethane to a flask containing lobelinic acid. The mixture was stirred and cooled. When all of the acid had reacted, the ether was removed by distillation and the residue was washed in a separatory funnel with water. The ester was removed and the water extracted with ether. The resulting ethereal solution of the ester was dried, the ether removed and the ester distilled as before. The yield was 80% of theory.

Pseudopelletierine.—Dry xylene (100 cc.) was placed in a 500-cc. 3-necked flask, fitted with a reflux condenser, mercury sealed stirrer and a dropping funnel. The top of the condenser was protected from moisture with a calcium chloride tube. Metallic sodium (1.38 Gm.) and 6 drops of absolute ethanol were added to the flask and the mixture heated until the sodium melted and the xylene boiled. While stirring rapidly, 16.5 Gm. of diethyl lobelinate, dissolved in 50 cc. of xylene, was added through the dropping funnel at such a rate that refluxing was maintained. After the addition of the ester, the flask was heated for an additional time (total time, two hours). The contents of the flask were allowed to cool and poured on a mixture of 50 Gm. of ice and 100 cc. of water. After stirring, the layers were allowed to separate and the xylene was extracted to completion with water. The combined aqueous extracts were cooled to 10° and acidified with hydrochloric acid. The cooled acidic solution was then alkalized with sodium carbonate and extracted with chloroform. The chloroform was removed by distillation and the residue was taken up in 200 cc. of 18% hydrochloric acid and the acidified solution boiled under reflux until a test sample gave no color change with ferric chloride solution.

The acid solution was alkalized with sodium carbonate and extracted with chloroform. The chloroform was removed by distillation and the residue was taken up with ether. The ethereal solution was allowed to evaporate at room temperature and then placed in a vacuum desiccator for three hours. A dark brown crystalline mass resulted which was dissolved in petroleum ether and filtered. On evaporation of the petroleum ether, 4.5 Gm. (50%) of pseudopelletierine having a slightly yellow color was obtained, m.p. 45°. Tanret (25) reported a melting point of 46° whereas Cope (5) reported the melting point of the hemihydrate of pseudopelletierine to be 48° and the anhydrous product, prepared by sublimation, to have a melting point of 64–65°. The product obtained here after sublimation also had a melting point of 64–65°.

Anal.—Calcd. for $C_9H_{15}NO$: C, 70.6; H, 9.9. Found: C, 69.7; H, 9.9.

The analytical results were substantially in agreement with the values calculated for pseudopelletierine. The low carbon value was undoubtedly due to the hygroscopic nature of the product. In order to eliminate any doubt, however, several derivatives were made and the melting points compared with those recorded in the literature. The following table shows these comparisons.

Derivative	M. p. (observed)	M. p. (literature)
Oxime	126–127°	128–129° (26)
Picrate	239–240° (decomp.)	252–253° (decomp.) (27)
Dibenzylidene	198–199°	200° (28)
Dipiperonylidene	225–226°	220–227° (1)

A known sample of pseudopelletierine, received from Dr. A. C. Cope of the Massachusetts Institute of Technology, showed no melting point depression when mixed with the product obtained in this work. Likewise, no depression of melting point was noted when mixed melts of the dibenzylidene and dipiperonylidene derivatives prepared from the two samples were taken.

SUMMARY

1. The reaction of dipicolinic acid dichloride with diazomethane was studied. The Wolff rearrangement of the diazoketone obtained could not be effected.

2. Two piperidine derivatives, believed to be 6-(2-picolyl)acetic acid ethyl ester and 6-(2-picolyl)benzoyl methane were prepared in a study of the reaction of 2,6-lutidine with phenyl lithium.

3. The preparation of pyridyl-2,6-diacetic acid diethyl ester hydrochloride is reported.

4. Norlobelinic acid, a new compound, was prepared along with the anilide and the dimethyl and diethyl esters of the acid.

5. A complete synthesis of lobelinic acid is reported and the dimethyl and diethyl esters prepared.

6. Pseudopelletierine was prepared by an inner acetoacetic ester condensation of diethyl lobelinate, followed by decarboxylation of the in-

termediate carbethoxypseudopelletierine. The yields of pseudopelletierine from diethyl lobelinate compare favorably with those from glutaraldehyde but the process also suffers because of the lengthy synthesis of diethyl lobelinate. Procedures to overcome this difficulty have as yet met with little success.

REFERENCES

(1) Menzies, R., and Robinson, R., *J. Chem. Soc.*, **1924**, 2163.
(2) Robinson, R., *ibid.*, **1917**, 762.
(3) Schöpf, C., and Lehman, G., *Ann.*, **518**, 1(1935).
(4) Ziegler, K., and Wilms, H., *ibid.*, **567**, 31(1950).
(5) Cope, A. C., *et al.*, *J. Am. Chem. Soc.*, **73**, 3416(1951).
(6) Willstätter, R., *Ann.*, **422**, 15(1921).
(7) Soine, T. O., and Buchdahl, M. R., THIS JOURNAL, **39**, 421(1950).
(8) Andersson, N., and Soine, T. O., *ibid.*, p. 460.
(9) Wieland, H., and Dragendorff, O., *Ann.*, **473**, 83 (1929).
(10) Adams, R., *et al.*, "Organic Reactions," Vol. I, John Wiley & Sons, Inc., New York, 1942, p. 38.
(11) Oparina, M. P., *J. Gen. Chem.* (USSR), **5**, 1699 (1935).
(12) Woodward, R. B., and Kornfeld, E. D., "Organic Syntheses," Vol. 29, John Wiley & Sons, Inc., New York, 1949, p. 44.
(13) Bergman, E. D., and Pinchas, S., *J. Org. Chem.*, **15**, 1186(1950).
(14) Kloppenburg, J. C., and Wibaut, J. P., *Rec. trav. chim.*, **65**, 393(1946).
(15) Sixma, F. L., and de Jong, J. I., *ibid.*, **69**, 547(1950).
(16) Barnes, R. A., and Fales, H. M., *J. Am. Chem. Soc.*, **75**, 3830(1953).
(17) Scheuing, C., and Winterhalder, L., *Ann.*, **473**, 126(1929).
(18) Chiang, M., and Hartung, W., *J. Org. Chem.*, **10**, 21 (1945).
(19) Blout, E., and Eager, V., *J. Am. Chem. Soc.*, **67**, 1315(1945).
(20) Wibaut, J. P., *et al.*, *Rec. trav. chim.*, **64**, 30(1945).
(21) Schuster, F., *Ber.*, **25**, 2398(1892).
(22) Shaw, B. D., *J. Chem. Soc.*, **1924**, 2363.
(23) Clarke, H. J., *et al.*, *J. Am. Chem. Soc.*, **55**, 4571 (1933).
(24) Fuson, R. C., *et al.*, *ibid.*, **68**, 1239(1946).
(25) Tanret, C., *Compt. rend.*, **86**, 1270(1878).
(26) Ciamician, G., and Silber, P., *Ber.*, **26**, 156(1893).
(27) *Ibid.*, **25**, 1601(1892).
(28) Piccinini, A., *Gass. chim. ital.*, **29**, I, 408(1899). Through *J. Chem. Soc.* **1899**, 829.

DDT vs. Wildlife*

Relationships Between Quantities Ingested, Toxic Effects and Tissue Storage

By JAMES B. DeWITT, JAMES V. DERBY, Jr., and GEORGE F. MANGAN, Jr.

Young and adult quail and pheasants were fed diets containing DDT. Chemical analysis of tissues indicated that quantities of DDT stored were not proportional to total amounts administered nor to duration of exposure, but were related to the severity of toxic symptoms. Concentration of the toxicant in breast muscle of birds dying of acute DDT poisoning was approximately 34 μg./Gm. in adult quail and 22 μg./Gm. in adult pheasants.

VARIOUS STUDIES on the effects of DDT upon wildlife have shown that significant reductions in avian populations have followed heavy or repeated applications of this insecticide (Benton, 1951, Hotchkiss and Pough, 1946, Mitchell, *et al.*, 1953, Stewart, *et al.*, 1946). Some of the observed decreases in bird numbers may have been due to mass movements out of treated areas following destruction of food insects, but Robbins and Stewart (1949) and Linduska and Surber (1948) cite instances of heavy mortality attributed to DDT intoxication.

Evidence that DDT was directly responsible for death of birds in treated areas has been available only in cases where typical symptoms were observed in dying individuals. Chemical analysis of carcasses collected from sprayed areas has been utilized to establish the presence of the toxicant in body tissues (Mitchell, *et al.*, 1953), but interpretation of resulting data has been difficult. Continued ingestion of sublethal quantities of DDT results in accumulation of the compound in body tissues (Laug, *et al.*, 1946), and this stored material may exert no toxic effects during periods of normal food intake. In the absence of data relating tissue storage to total quantity ingested, rate of ingestion, or severity of toxic symptoms, the presence of DDT in tissues merely establishes that the bird or animal had at some time ingested food contaminated with the compound.

Studies designed to furnish information on relationships between quantities of DDT ingested, toxic effects, and tissue storage in avian species were conducted at the Patuxent Research Refuge during 1950–1953. Measured quantities of the toxicant were administered orally, or were incorporated in diets fed young and adult birds. Chemical analyses were made to determine concentrations of DDT in tissues in cases of acute and sublethal poisoning.

EXPERIMENTAL PROCEDURES

Quail and Pheasant Studies.—Groups of young and adult quail and pheasants, obtained by random

* Received August 6, 1954 from the U. S. Department of the Interior, Fish and Wildlife Service, Laurel, Md. Part of the data in this paper were taken from a thesis submitted by George F. Mangan, Jr., in partial fulfillment of the requirements for the degree of Master of Science, Georgetown University, 1954.

selection from pen-reared stock, were given *ad libitum* access to diets containing the following percentages of DDT:

Adult quail—0.025, 0.050, 0.100, 0.200%
Young quail—0.005, 0.010, 0.025, 0.050, 0.100%
Adult pheasants—0.025, 0.050, 0.100, 0.200, 0.400%
Young pheasants—0.005, 0.010, 0.025%

The young birds were placed on the experiment immediately following their removal from the hatchers, while the adult birds were approximately one year old at the start of the test. Records were kept on food consumption, body weights, onset of toxic symptoms and mortality. Carcasses of all birds dying during the experiment, or sacrificed at the end of the study, were stored at 4° F. pending chemical analysis.

Starling (Nestlings) Studies.—Twenty-two broods involving 89 birds were included in these studies. In all cases, the young birds remained in the nests and were undisturbed except for daily weighing and dosing. One bird in each nest was designated as a control and received no DDT. All others were given daily doses of an aqueous suspension of DDT in gum acacia at levels of 25, 50, 75, 100, 150, or 200 mg./Kg. of body weight. At the end of the five-day test period, surviving birds were killed, or were deprived of food until death resulted from starvation or from DDT intoxication. All carcasses were held at 4° F. pending analysis.

Laboratory Procedures.—Carcasses were allowed to thaw, and feathers, feet, wing tips, and beaks were removed and discarded. In the case of very small birds, the entire remaining carcass was passed through a meat grinder and analyzed. With larger birds, the gastrointestinal tract and a section of breast muscle were removed and analyzed separately.

The finely divided tissues were placed in a Waring Blendor and extracted for ten minutes with a mixture of acetone and petroleum ether (67.8% acetone, 32.2% petroleum ether). The extraction mixture was filtered under suction, and the residue re-extracted with fresh solvent. An additional extraction with diethyl ether was made, if necessary, to obtain a white, powdery residue. The combined extracts were evaporated to dryness in an air stream over a steam bath, dissolved in carbon tetrachloride, and analyzed for DDT according to procedures prescribed by the Association of Official Agricultural Chemists (1950).

RESULTS AND DISCUSSION

The results of these studies are summarized in Tables I and II. Data presented in these tables show quantities of DDT ingested or administered,

TABLE I.—EFFECTS UPON QUAIL AND PHEASANTS OF DIETS CONTAINING DDT

Species	DDT Content of Diet, %	Mortality, %	Survival Time, days		DDT Ingested, mg./Kg.				DDT Content of Tissues, μg./Gm.		
					Daily		Total				
			Av.	Range	Av.	Range	Av.	Range	Muscle	G.I.[a]	Entire Carcass
Quail, Adult	0.025	100.0	45.0	29–63	24.5	...	1,100	700–1,550	32.0	61.3	...
	0.050	100.0	33.0	29–39	45.2	...	1,490	1,310–1,760	39.0	38.4	...
	0.100	100.0	11.0	6–14	102.0	...	1,120	610–1,430	34.7	89.7	...
	0.200	100.0	7.0	4–9	318.0	...	2,225	1,270–2,860	32.5	42.5	...
Quail, Young	0.005	23.0	...	12–	8.1	5.1–145	530	145–	...	...	1.6
	0.010	8.4	...	22–	14.0	10.4–26.6	975	510–	...	...	4.1
	0.025	10.0	...	22–	43.8	26.1–71.1	3,065	1,345–	...	...	19.3
	0.050	100.0	33.2	20–49	88.3	54.4–96.4	3,710	1,855–	...	...	40.8
	0.100	100.0	10.0	3–20	130.0	123.0–135.0	2,725	370–	...	...	73.2
Pheasant, Adult	0.025	100.0	18.2	11–24	11.6	...	205	125–280	18.8	131.3	...
	0.050	100.0	15.0	6–20	20.0	...	309	120–400	24.9	212.0	...
	0.100	100.0	5.3	2–9	50.8	...	269	100–460	11.3	57.8	...
	0.200	100.0	4.8	4–6	42.1	...	202	170–380	33.3	50.4	...
	0.400	100.0	3.4	2–6	60.5	...	206	120–360	30.0	76.9	...
Pheasant, Young	0.005	42.8	...	9–	11.3	...	...	...	3.6	12.0	...
	0.010	100.0	27.3	7–51	21.2	...	615	150–1,080	25.2	18.7	...
	0.025	100.0	24.8	4–41	49.7	...	1,120	200–2,000	...	...	100.3

[a] Gastrointestinal tract.

TABLE II.—EFFECTS OF DDT ON STARLING NESTLINGS

Dosage Level, mg./Kg.	Mortality, %	Total DDT Admin. Av., mg.	DDT Content of Tissues							
			Group A[a]		Group B[a]		Group C[a]		Group D[a]	
			Body	G.I.[b]	Body	G.I.	Body	G.I.	Body	G.I.
0	17.6	0.0	1.2	0.0	...	...	1.3	8.1	1.1	3.6
25	10.0	5.19	66.0	102.7	30.9	54.2	29.8	...	34.5	41.2
50	18.2	9.02	64.3	82.6	53.0	84.2	38.6	58.5	67.2	64.8
75	20.0	11.71	59.2	...	64.1	108.6	...	...	43.7	53.5
100	16.7	21.25	92.9	116.0	67.6	81.7	63.4	67.8	57.5	120.4
200	71.4	39.43	71.2	135.7	51.3	145.6	84.0	119.8	...	...
		Av.	72.3	117.8	56.1	90.9	53.0	74.5	52.3	71.8

[a] Group A—Birds dying during experimental period. Group B—Birds developing symptoms of DDT poisoning after periods of starvation. Group C—Birds dying after periods of starvation, without visible symptoms of DDT poisoning. Group D—Birds which were killed at the end of the experiment.
[b] Gastrointestinal tract.

percentage mortality, survival times, and amounts of the toxicant found in body tissues.

Typical symptoms of DDT poisoning resulted when adult quail were fed diets containing 0.025–0.200% of the compound. At all levels, the birds lost 15–30% body weight, and developed tremors and muscular incoordination twenty-four to forty-eight hours before death. Time required for development of these symptoms was inversely proportional to concentration of DDT in the diets, and was related to total amounts of the poison ingested. The minimum lethal quantity was 610 mg./Kg., and more than 50% of the birds died after ingesting less than 1,500 mg./Kg. Quantities of DDT stored in muscle tissues were not directly proportional to total amounts ingested, nor to duration of feeding, and ranged from 20 to 45 μg. per gram of tissue. The data indicate that the presence of 30–40 μg. DDT per gram of breast muscle would be presumptive evidence that adult quail had ingested a lethal quantity of the toxicant.

Although adult quail were unable to survive on diets containing 0.025% DDT, young quail apparently were unharmed when fed at this level during the first ten weeks of life. Ninety per cent of the birds receiving this diet survived after ingesting more than 3000 mg. DDT/Kg. body weight, with daily intakes ranging from 26 to 71 mg./Kg. Daily ingestion of 90–125 mg./Kg. resulted in typical symptoms of DDT poisoning. Tissue storage was not proportional to total quantities consumed, nor to rate of daily intake.

Pheasants apparently are more susceptible than quail to the effects of DDT. Maximum survival time for adult pheasants fed diets containing 0.025 per cent of the compound was twenty-four days, whereas quail survived for appreciably longer periods. Some birds died after ingesting as little as 100 mg./Kg., and the maximum quantity consumed by any adult pheasant was less than 500 mg./Kg. Tissue storage ranged from 11 to 53 μg. per gram of muscle tissue. The presence of more than 15–25 μg./Gm. in breast muscle of adult pheasants would indicate that the birds had ingested lethal quantities of DDT.

Young pheasants were able to tolerate a level of 0.005% DDT in the diet for a period of six weeks before excessive mortality occurred. Levels of 0.01 and 0.025% were highly toxic, and all birds receiving these diets died within seven weeks. Ingestion of as little as 200 mg./Kg. was lethal to some birds, although others consumed more than 2,000 mg./Kg. before succumbing to the effects of DDT. This, as in the case of quail, indicates wide variation in susceptibility among individuals of the same species. DDT was stored at all levels fed, and the presence of more than 25 μg./Gm. in breast muscle would be indicative that young pheasants had succumbed to the effects of the insecticide.

Although experimental conditions were not directly comparable, starling nestlings appeared more resistant than quail or pheasants to the effects of DDT. Mortality among birds receiving 5 daily doses of 100 mg./Kg. was not significantly different from that of undosed nest mates, and some birds survived after receiving 5 doses of 200 mg./Kg. Other young starlings showed typical symptoms of DDT poisoning and died after ingesting 2 doses of 50 mg./Kg. Storage of DDT in body tissues was not directly proportional to quantities administered, duration of exposure, nor to severity of symptoms of poisoning.

SUMMARY AND CONCLUSIONS

1. Typical symptoms of DDT poisoning resulted when quail and pheasants were fed diets containing 0.025–0.050 per cent of the compound. Time required for development of toxic symptoms decreased with increases in percentages of DDT in the diets, and young birds appeared more resistant than adults.

2. Median lethal quantity for adult quail was approximately 1,200 mg./Kg., but young quail showed no ill-effects after ingesting 3,000 mg./Kg. during a ten-week period.

3. Adult pheasants succumbed after ingesting 200–300 mg./Mg., while approximately 600 mg./Kg. were required to kill young birds.

4. Chemical analysis of tissues indicated that quantities of DDT stored were not proportional to total amounts administered nor to duration of exposure, but were related to the severity of toxic symptoms. Concentration of the toxicant in breast muscle of birds dying of acute DDT poisoning was approximately 34 μg./Gm. in adult quail and 22 μg./Gm. in adult pheasants.

REFERENCES

(1) "Official Methods of Analysis," Assoc. Offic. Agr. Chemists, 1950, 910 pp.
(2) Benton, A. H., *J. Wildl. Mgt.*, **15**, 20(1951).
(3) Hotchkiss, N., and Pough, R. H., *ibid.*, **10**, 202(1946).
(4) Laug, E. P., and Fitzhugh, O. G., *J. Pharmacol. Exptl. Therap.*, **87**, 18(1946).
(5) Linduska, J. P., and Surber, E. W., "Effects of DDT and Other Insecticides on Fish and Wildlife," Circular No. 15, U. S. Dept. Interior, Fish and Wildlife Service, 1948, 19 pp.
(6) Mitchell, R. T., Blagbrough, H. P., and VanEtten, R. C., *J. Wildl. Mgt.*, **17**, 45(1953).
(7) Robbins, C. S., and Stewart, R. E., *ibid.*, **13**, 11 (1949).
(8) Robbins, C. S., Springer, P. F., and Webster, C. G., *ibid.*, **15**, 213(1951).
(9) Stewart, R. T., Cope, J. B., Robbins, C. S., and Brainerd, J. W., *ibid.*, **10**, 195(1946).

Buffered Ophthalmic Solutions of Silver Nitrate*

By WALTER F. CHARNICKI and MARY L. KOBER

One per cent silver nitrate solutions have been prepared in an acetate buffer at *p*H 6.0 and have been shown to remain stable for as long as two years. A simple colorimetric method using bromcresol purple as the indicator has been used to detect increased acidity in the solutions. This dye is especially sensitive in the range of *p*H 5.8 to 6.0, the uppermost limits specified by the U. S. P. XIV for Silver Nitrate Ophthalmic Solution. Preliminary pharmacological investigations have shown no appreciable differences between an official solution and one with a *p*H below the U. S. P. XIV specification.

SOLUTIONS of silver nitrate, one per cent, are commonly used for the prevention of ophthalmia neonatorum in new born infants, and in many states this prophylactic treatment is required by law. Commercial preparations for this purpose are customarily supplied in the form of wax ampuls, from which a drop may be instilled into the conjunctival sac of the eye. To minimize the irritating action of the silver nitrate, the U. S. P. XIV designates that Silver Nitrate Ophthalmic Solution be between *p*H 5 and 6. Unbuffered solutions or solutions whose hydrogen-ion concentrations are merely adjusted between *p*H 5 and 6 show a progressive drop in *p*H upon aging so that buffers should be used. This drop in *p*H is accelerated if beeswax is included as an ingredient in the wax ampul (1). The *p*H determinations of silver nitrate solutions using a standard *p*H meter equipped with glass-calomel electrodes can be obtained only with difficulty as the silver tends to coagulate at the tip of the junction. This, however, can be overcome with the use of a special type electrode or with a color indicator which is effective in the desired *p*H range. Since very little could be found in the literature regarding the buffering of silver nitrate solutions, this study is concerned with their preparation and stability.

EXPERIMENTAL

Determination of pH.—A Beckman Model H-2 *p*H meter equipped with glass-calomel electrodes was used for all determinations in the early part of the study. For solutions containing silver nitrate, readings were taken immediately upon immersing the electrodes. On many occasions this method was not satisfactory as the needle would oscillate over a wide range and made accurate readings difficult. This difficulty could be overcome with the use of a double-junction type electrode. As the study progressed, however, and there was no need for determining the precise measure of acidity below *p*H 5.0, the lower limit of the U. S. P. specification, it was decided to experiment with a colorimetric method. Since the solutions were to be buffered in the uppermost limit of the U. S. P. specification, namely *p*H 5.8–6.0, it was necessary to use an indicator effective within this range. Universal indicator (2) was prepared with the following composition: phenolphthalein 0.1 Gm., methyl red 0.2 Gm., dimethylaminoazobenzene 0.3 Gm., bromothymol blue 0.4 Gm., and thymol blue 0.5 Gm. in 500 ml. alcohol, with sufficient sodium hydroxide 0.1 *N* to produce a yellow color. The indicator had a range of cherry-red at *p*H 1.0 to blue at *p*H 10.0, and was first tried on a series of standards prepared with sodium acetate-acetic acid solutions which ranged from *p*H 5.2 to 7.0 at 0.2 *p*H intervals. This indicator was unsatisfactory in that it could not distinguish between *p*H 5.8 and 6.2. Bromcresol purple (3) (dibromo-*o*-cresolsulfonphthalein), with a range of yellow at *p*H 5.2 to purple at *p*H 6.8, was tested in a similar manner. Two drops of indicator solution added to 10 ml. of buffer solution produced the results found in Table I. The bromcresol indicator was the colorimetric method of choice, and it was used for *p*H determinations in the latter part of the study.

TABLE I.—COLOR REACTIONS WITH BROMCRESOL PURPLE INDICATOR

*p*H Buffer Solution	Resulting Color Reaction with Indicator
5.2	Bright yellow
5.4	Faint yellow
5.6	Faint yellow
5.8	Almost colorless
6.0	Purple
6.2	Purple

Effect of Buffering.—Three general types of solutions were made: *Unbuffered solutions.*—One per cent solutions of silver nitrate were prepared in cold distilled water with agitation. The resulting *p*H was 5.85. *Adjusted solutions.*—The unbuffered solutions were raised from *p*H 5.85 to *p*H 5.9–6.0 by the addition of a sufficient quantity of 0.02 *N* sodium hydroxide solution. *Buffered solutions.*—Buffer mixtures at *p*H 6.1 were prepared from 0.5% sodium acetate and 0.1% glacial acetic acid solutions. This required sodium acetate, 0.06 mole and acetic acid, 0.0166 mole, respectively. One per cent silver nitrate was added to the buffer mixtures, which lowered the *p*H to 6.0–6.05.

The above solutions were put into amber-colored bottles which had been coated on the inside with various waxes to simulate the commercial wax ampuls. One lot of bottles was coated with paraffin U. S. P., a second lot with yellow wax U. S. P., a

* Received May 1, 1954, from the Research Division Laboratories, Sharp and Dohme Division, Merck and Co., Inc., West Point, Pa.

TABLE II.—A COMPARISON OF THE *p*H OF UNBUFFERED, ADJUSTED, AND BUFFERED SOLUTIONS OF ONE PER CENT SILVER NITRATE

		Type Coated Containers							
		Paraffin		Yellow Wax		Superla® Wax Yellow		Ceresin Wax White	
Solution	Age	Room Temp.	50°	Room Temp.	50°	Room Temp.	50°	Room Temp.	50°
Unbuffered	Initial	5.85	5.85	5.85	5.85				
	2 weeks	5.25	5.52	3.28	2.52				
	1 month	5.00[a]	5.00[a]	3.00[a]	2.00[a]				
	2 months	4.30	4.80	2.85	2.40				
	3 months	4.75	4.61	2.72	2.20				
	6 months	<5.20	<5.20	<5.20	<5.20				
	18 months	<5.20	<5.20	<5.20	<5.20				
	24 months	<5.20	<5.20	<5.20	<5.20				
Adjusted 0.02 *N* Sodium Hydroxide	Initial	6.00	6.00	6.00	6.00	5.90	5.90	5.90	5.90
	2 weeks	5.50	4.78	3.28	2.51	5.30	4.60	5.60	5.10
	1 month	5.00[a]	5.00[a]	3.00[a]	2.00[a]	4.99	4.42	5.55	4.98
	2 months	5.00	4.30	2.82	2.35	<5.20	<5.20	5.40	<5.20
	3 months	4.75	3.90	2.85	2.10	<5.20	<5.20	<5.20	<5.20
	6 months	<5.20	<5.20	<5.20	<5.20	<5.20	<5.20	<5.20	<5.20
	18 months	<5.20	<5.20	<5.20	<5.20	<5.20	<5.20	<5.20	<5.20
	24 months	<5.20	<5.20	<5.20	<5.20	<5.20	<5.20	<5.20	<5.20
Buffered Acetate-Acetic Acid	Initial	6.05	6.05	6.05	6.05	6.00	6.00	6.00	6.00
	2 weeks	6.05	6.05	5.70	4.80	5.95	5.92	5.95	5.95
	1 month	5.00[a]	5.00[a]	5.00[a]	5.00[a]	6.00	6.05	6.02	6.08
	2 months	6.10	6.09	5.38	4.25	6.00	6.00	6.00	6.00
	3 months	6.05	5.75	5.20	3.80	6.00	6.00	6.00	6.00
	6 months	6.00	6.00	<5.20	<5.20	6.00	6.00	6.00	6.00
	9 months	6.00	6.00	<5.20	<5.20	6.00	6.00	6.00	6.00
	18 months	6.00	6.00	<5.20	<5.20	6.00	6.00	6.00	6.00
	24 months	6.00	6.00	<5.20	<5.20	6.00	6.00	6.00	6.00

[a] These readings are very approximate as more trouble than usual was experienced with the *p*H meter.

third lot with Superla®[1] wax yellow, and a fourth lot with ceresin wax white. These waxes were selected because they have been used as ingredients in some of the commercial wax ampuls. The coated containers were placed at room temperature and 50° stability stations in order to determine the effect of these coatings upon the *p*H of the silver nitrate solutions. The results of these tests are shown in Table II.

The *p*H readings recorded in the table for the first three months of the paraffin and yellow wax studies and for the first month of the Superla® wax yellow and ceresin wax white studies (later experiments) were determined with the Beckman *p*H meter. All other *p*H determinations were obtained with bromcresol purple indicator.

Preliminary Acute Irritation Studies.[2]—An acute corneal irritation test was conducted on the buffered solution (*p*H 6.0), the buffer vehicle (*p*H 6.1), and the adjusted *p*H solution (*p*H 4.5). The adjusted solution was an aged sample whose *p*H had dropped below the lower limit of the U. S. P. specification. These solutions were submitted for a preliminary comparison of their ability to produce irritation in the rabbit's eye. The procedure was that of standard silver nitrate application with saline washout. The rabbits were observed until all irritation subsided. Results indicated no apparent difference in the degree of irritation between the silver nitrate preparations. In the rabbit's eye these solutions caused a marked injection and chemosis of the conjunctivae. This irritation subsided after forty-eight hours. When the treated eyes were examined at seven days, all evidence of irritation was absent. The acetate buffer solution alone caused only minimal injection of the conjunctivae immediately following instillation. This effect was transient.

DISCUSSION

The choice of buffers was limited to those which would form soluble compounds with the silver ion; therefore, buffer systems effective within the desired range could not be used. Acetate buffer, which meets the solubility requirement, exhibits its maximum buffer capacity in the neighborhood of *p*H 4.6–4.7. At the desired *p*H (6.0) the buffer capacity of acetate is approximately one-eighth that at *p*H 4.7. In view of this limited capacity it was necessary to determine whether acetate could be used for this purpose. Our results have shown that acetate is capable of buffering one per cent silver nitrate solutions at a *p*H of 6.0 for as long as two years.

The colorimetric method using bromcresol purple as the indicator provided a rapid means of detecting an increase in acidity in the silver nitrate solutions prepared at a *p*H of 6.0. This indicator is especially sensitive for showing changes between *p*H 5.8 and 6.0, the range with which we were concerned in the latter part of our study. Bromcresol purple, however, cannot differentiate between hydrogen-ion concentrations of less than *p*H 5.2.

The *p*H of both unbuffered and adjusted (*p*H raised with sodium hydroxide) solutions dropped on standing at room temperature and 50° in all of the wax-coated containers. Beeswax not only accelerated this drop, but also caused a change in the *p*H of buffered solutions. This was due probably to an organic acid contained in the beeswax which would suggest the following reaction:

[1] Registered trademark of Standard Oil Company (Indiana).

[2] The authors are indebted to Mr. S. E. McKinney and Mr. H. L. McCrorey for the pharmacological data.

$$RCOOH + AgNO_3 \rightarrow RCOOAg + HNO_3$$

In this study nothing was done toward isolating or identifying the substance responsible for this increased acidity.

The preliminary pharmacological studies did not show any appreciable difference in irritation between a solution of 1% silver nitrate whose *p*H was within the upper limit of the U. S. P. specification (*p*H 6.0) and one whose *p*H was below the U. S. P. specification (*p*H 4.5). It is quite likely that silver nitrate, a known caustic agent, would be irritating regardless of *p*H. Nevertheless, ophthalmic solutions are made generally to be as isohydric as possible with the lacrimal fluid of the eye. This is, undoubtedly, the intent of the U. S. P. XIV regarding Silver Nitrate Ophthalmic Solution and prompted our investigations.

SUMMARY

1. One per cent solutions of silver nitrate have been buffered at *p*H 6.0.

2. A simple and rapid colorimetric method has been used to detect a drop in *p*H of the solutions.

3. Preliminary pharmacological studies have not shown appreciable differences in irritation between solutions of one per cent silver nitrate at *p*H 6.0 and *p*H 4.5

REFERENCES

(1) Wilkie, W. (to Parke, Davis and Co.), U. S. pat. 1,527,659, Feb. 24, 1925.

(2) "The Merck Index," 5th ed., Merck and Co., Inc., Rahway, N. J., 1940, p. 601.

(3) *Ibid.*, p. 598.

The Chronic Oral Toxicology of the Polyethylene Glycols*

By HENRY F. SMYTH, Jr., CHARLES P. CARPENTER, and CARROL S. WEIL

Polyethylene glycols were fed in the diet to rats for two years and to dogs for one year. Apparently a compound with a mean molecular weight of 6,000 is definitely less toxic by mouth than those of lower molecular weights. This work supports the view that the polyethylene glycols may be considered inert when taken by mouth.

POLYETHYLENE glycols of various mean molecular weights are now widely used in a variety of cosmetics and pharmaceuticals. Those of mean molecular weights of 400 and 4000 are described by monographs in the Fourteenth Revision of the U. S. Pharmacopeia and those of 300, 1500, 1540, and 4000 mean molecular weights have been included in New and Nonofficial Remedies for several years. The most recent review of the toxicology of the polyethylene glycols was published by the present authors in 1950 (1). That paper presented reason to believe that there had been a reduction in the chronic toxicity of the materials produced commercially after 1941, the year of manufacture of the samples which had been most thoroughly studied toxicologically. Since that publication studies have been completed which support the view of improvement and which give reason to disregard quantitative toxicological conclusions of the 1947 article by Smyth, Carpenter, and Shaffer (2) on the ground that new work does not duplicate their findings, perhaps because their samples are no longer typical of commercial production.

Throughout the rest of the present paper the polyethylene glycols (sometimes known as polyoxyethylenes) will be referred to in abbreviated form as PEG compounds. PEG 200, 300, 400, and 600 are fluids; 1000, 1540, 4000, and 6000 are solids of increasing firmness, and PEG 1500 is a solid blend of PEG 300 and 1540 in substantially equal weights. The number shows the approximate mean molecular weight of the molecules in a particular product, the individual molecules following a distribution of molecular weights approximating Flory's theories on linear condensations of ethylene oxide. The general chemical formula representing any PEG may be written in a variety of ways. One form is shown below:

$$HOCH_2CH_2(OCH_2CH_2)_nOH$$

PEG 200 is the lowest member of the series to be generally considered a polyethylene glycol. With PEG 200 n is approximately equal to 3.

SUBACUTE ORAL DOSES IN RATS

In order to determine whether there is a systematic change in quantitative toxicity of PEG compounds with increasing mean molecular weight, samples of nine commercial products (PEG 200, 300, 400, 600, 1000, 1500, 1540, 4000, and 6000) were

* Received August 27, 1954 from the Mellon Institute of Industrial Research, University of Pittsburgh, Pittsburgh 13, Pa.

The polyethylene glycols discussed in this paper are commercial products of Carbide & Carbon Chemicals Company, a unit of Union Carbide & Carbon Corporation, and they are sold under the trademark name of "Carbowax" compounds.

obtained, all produced within a three-week period late in 1950. Each of these was administered in the diet to five male and five female rats at levels of 2, 4, 8, 16, and 24% with a control group receiving no polyethylene glycol, using the methods described in more detail in the section on chronic toxicity. During a 90-day period the following criteria of effect were studied: mortality, appetite, body weight both in grams and as a ratio to the amount of nutrient consumed, liver weight, kidney weight, and micropathology of liver and kidney. Table I indicates the effects found at the different dosages, using a 1 in 19 probability as a level of significance.

TABLE I.—SUMMARY OF EFFECTS FROM 90-DAYS' FEEDING TO RATS OF DIETS CONTAINING POLYETHYLENE GLYCOLS[a]

Mean Molecular Weight	Concentration in the Diet— 2%	4%	8%	16%	24%
200	O	O	O	L	KLN
300	O	O	W	KL	LNW
400	O	O	O	W	KL
600	O	O	O	KW	KW
1,000	O	O	O	W	W
1,500	O	O	W	W	KW
1,540	O	O	W	W	KW
4,000	O	O	W	KW	KW
6,000	O	O	O	O	KW

[a] Meaning of symbols: O = no effect noted; N = decrease in nutrient eaten; W = decrease in weight gain of either sex in terms of grams per 100 Gm. nutrient eaten; L = liver heavier than that of control rats; K = kidney heavier than that of control rats.

With the exception of PEG 9000 the differences between products appear no greater than might be expected in studies of successive lots of the same product. Between a mean molecular weight of 200 and one of 4000 there was not found any relationship between molecular weight and subacute toxicity to rats, the eight products in this range appearing to give indistinguishable results. Apparently PEG 6000 is definitely less toxic than those of lower molecular weight.

CHRONIC TOXICITY TO RATS

PEG 1540 produced in 1949 and PEG 400 U. S. P. and 4000 U. S. P. produced in 1952 were fed in the diet of rats for two-year periods. The first material was fed to Sherman strain rats raised in our own colony, 35 of each sex in each dosage group (0, 0.02, 0.08, 0.4, 2, 4, and 8%) with the 56th day of age the first day on the diets. The latter two materials were fed to purchased Wistar-derived rats, 20 of each sex in each dosage group (0, 1, 2, 4, and 8% for PEG 400 and 0, 0.5, 1, 2, 4, and 8% for PEG 4000) with the 64th day of age the first day on the diet. The animals were kept five in a wire-mesh cage, the sexes separated. Food and water were available at all times.

The diet consisted of 60 parts of freshly ground whole wheat, 30.5 parts of dried whole milk, 1.5 parts of dried liver extract U. S. P., 5.0 parts dried inactive brewers' yeast, 1 part calcium carbonate, and 2 parts iodized table salt. With PEG 400 and 4000 the diet was changed from the 7th to the 14th month to a commercial rat pellet ground in our laboratory in order to reduce routine labor and expense. Calculation showed the calorific value of the two diets to be identical. Figure 1 shows that the rats grew less rapidly and ate more of the commercial diet and explains why the original diet was restored. The diets described were mixed with the PEG compounds to give the concentrations listed in the paragraph above.

During the study, food consumption, body weight, and blood cytology were followed. Mortality and extraneous infections were recorded. Gross and often micropathology were studied on animals dying from any cause. After a year of doses, a total of 125 animals were sacrificed, liver and kidney weights were determined, and micropathology was studied on adrenal, heart, small intestine, kidney, liver, lung, pancreas, spleen, and testis. After two years of doses the survivors were sacrificed and liver and kidney weights were determined. For PEG 1540, micropathology was also studied at this time. A total of 890 rats was used in the study and 141 were alive at twenty-six months of age after two years of doses. This is a survival of 17% of those not sacrificed for early study, about midway in the range of mortalities Barnes and Denz (3) have reported for similar recent studies described in the literature. Almost 90% of the deaths were due to pneumonia, equally distributed between those receiving PEG compounds and the controls.

Only minor effects were found. At 8% PEG 1540 there was somewhat more light cloudy swelling of the liver in treated than in control rats, at 4% and 8% PEG 400 male rats grew slightly less than did the male controls, and at 8% PEG 4000 both male and female rats grew somewhat less than did their controls. In all other observations made, the rats receiving PEG compounds differed from their specific controls no more than could be due to random variations, using statistical methods with a 1 in 19 level of significance and considering the sexes both together and separately.

CHRONIC TOXICITY TO DOGS

PEG 400 U. S. P. produced in 1952 and PEG 1540 and 4000 produced in 1950 were fed in the diet of dogs for one-year periods.

For PEG 400 each group consisted of 4 dogs, one male foxhound ten months old, one male and one female Doberman Pinscher by Kerry Blue Terrier cross eight months old, and one male Beagle sixteen or twenty-three months old. One group was fed a commercial dog food meal wetted with a solution of PEG 400 equivalent to 2% PEG in the diet. The second group received the same meal wetted with water only.

In advance of starting one-year doses of PEG 1540 and 4000 two mongrel adult male dogs were started as a pilot experiment to verify diet acceptance. For four weeks they were given 2% PEG compounds in their diet and were then raised to 8%. Since no effect was noticed these two dogs were continued to one year of dosing. The main study consisted of three groups of four dogs each taken at random from 6 male Beagles and 3 male and 3 female Cocker Spaniels 16 to 22 months old. One group was fed a commercial dog food meal wetted with a solution of PEG 1540 equivalent to 2% PEG in the diet, the second with PEG 4000 and the third the same meal wetted with water only.

During the year body weight, blood cytology,

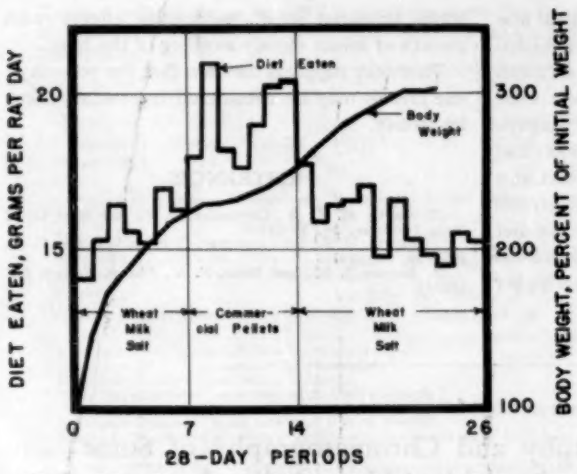


Fig. 1.—Mean diet eaten and body weight of control rats, showing effect of substituting commercial rat pellets for wheat milk salt mixture.

bromsulfalein retention, and prothrombin time were followed. For the first two materials plasma cholinesterase levels were also followed, and for the last serum urea nitrogen and alkaline serum phosphatase were estimated every three months. At the end of the year the dogs were sacrificed, gross pathology was carefully studied, including search for urinary and gall bladder concretions, and livers and kidneys were weighed. Micropathology was studied in adrenal, bladder, heart, kidney, liver, lung, pancreas, small intestine, stomach, spleen, thyroid, and testis or ovary.

Table II summarizes the initial weights and the quantitative observations made after a year of feeding. Where the entries consist of the means of observations upon 4 dogs the standard deviations are shown. None of the observations upon treated dogs differed from the means of their control groups using statistical methods with a 1 in 19 level of significance. The dogs receiving PEG 1540 and 4000 were older than those receiving PEG 400, hence neither dosed or control dogs changed much in weight in the year of study. The fact that those receiving 2% PEG 4000 actually lost a little weight is seen to have no significance both from the large standard deviation of the mean weight changes and from the fact that the dog on 8% of the same material gained weight in the same period.

No abnormalities were found in the gross pathology or micropathology of the organs named above and the quantitative measurements showed no difference between dogs receiving PEG compounds in their diets and their specific controls.

CONCLUSIONS

New chronic oral toxicity studies in animals confirm the inferences of our 1950 paper (1) that polyethylene glycols produced commercially since 1941 are much less toxic than the samples two of us first reported upon (2). During ninety days rats are affected so nearly identically by polyethylene glycols from a mean molecular weight of 200 up to one of 4000 that there seems to be no

TABLE II.—POLYETHYLENE GLYCOLS IN THE DIET OF DOGS FOR ONE YEAR (MEAN QUANTITATIVE OBSERVATIONS ± STANDARD DEVIATIONS)

	PEG 400		PEG 1540			PEG 4000	
	2% 4 dogs	Control 4 dogs	8% 1 dog	2% 4 dogs	Control 4 dogs	8% 1 dog	2% 4 dogs
Initial weight, Kg.	15.90 ± 3.60	16.01 ± 2.00	15.42	10.72 ± 1.54	10.76 ± 1.98	10.48	10.92 ± 2.90
Values after one year							
Weight Kg.	20.42 ± 4.86	21.06 ± 4.24	18.38	11.73 ± 1.25	11.04 ± 2.08	11.00	10.34 ± 2.65
Weight change from initial wt., Kg.	+4.52 ± 2.55	+5.04 ± 2.85	+2.96	+1.01 ± 0.60	+0.28 ± 0.76	+0.52	−0.58 ± 1.25
Liver weight, % body wt.	2.58 ± 0.10	3.13 ± 0.68	2.89	2.74 ± 0.29	.....	2.51	3.26 ± 0.32
Kidney weight, % body wt.	0.42 ± 0.04	0.46 ± 0.02	0.44	0.45 ± 0.10	.....	0.45	0.56 ± 0.10
Red blood cells, million/cu. mm.	7.02 ± 0.87	5.61 ± 0.38	6.25	6.67 ± 0.65	5.65 ± 0.38	8.46	6.32 ± 0.72
Hemoglobin, Gm./100 ml.	16.8 ± 0.5	16.9 ± 0.5	13.8	16.2 ± 0.8	14.6 ± 1.5	19.3	14.1 ± 0.7
Bromsulfalein retention, % at 15 min.	7.98 ± 2.09	6.55 ± 0.95	7.99	8.61 ± 3.18	10.10 ± 1.26	7.29	7.12 ± 2.08
Prothrombin time, min.	7.6 ± 0.4	7.6 ± 0.5	6.8	6.9 ± 0.8	6.8 ± 0.3	7.6	6.8 ± 1.2
Serum urea nitrogen, mg./100 ml.	17.50 ± 2.71	16.80 ± 3.18	..	.....	.....	..	.....
Alkaline serum phosphatase, mM units	1.54 ± 0.58	0.86 ± 0.28	..	.....	.....	..	.....
Plasma cholinesterase, micro 1.CO_2 30 min.	.....	.....	69.1	100.1 ± 20.7	121.4 ± 13.3	106.5	104.5 ± 20.8

justification for considering the 8 commercial materials in this range as toxicologically distinguishable. Apparently a compound with a mean molecular weight of 6000 is definitely less toxic by mouth than those of lower molecular weights.

Polyethylene glycols 400, 1540, and 4000 had no effect upon dogs when present in the diet at a level of 2% for one year. Polyethylene glycols 1540 and 4000 had no effect upon rats in the diet at 4% for two years, and polyethylene glycol 400 had no effects at 2% for two years. In rats higher levels produced small, nonspecific effects upon growth or minor cloudy swelling of the liver.

This work supports the view that the polyethylene glycols may be considered inert when taken by mouth.

REFERENCES

(1) Smyth, H. F., Jr., Carpenter, C. P., and Weil, C. S., THIS JOURNAL, 39, 349(1950).
(2) Smyth, H. F., Jr., Carpenter, C. P., and Shaffer, C. B., *ibid.*, 36, 157(1947).
(3) Barnes, J. M., and Denz, F. A., *Pharm. Rev.*, 6, 191 (1954).

A Note on Polarography and Chromatography of Some Commercially Available Chlorophyllins*

WALTER L. WUGGATZER† and JOHN E. CHRISTIAN

THE AUTHORS have presented in a preliminary report (1) the results of their investigation on the polarographic behavior of some commercially available chlorophyllins. Since the appearance of this report, ten additional commercial chlorophyllins have been made available for study. Eight of these were copper chlorophyllins, one was a cobalt chlorophyllin, and the last sample had no metal content.

Voigtlander and Henning (2) and Eddy (3) have pointed out that the chlorophyllins are mixtures of several components rather than being pure substances. Since the chlorophylls have been separated by adsorption chromatography (4) this method was believed applicable to the separation of the several components of the chlorpohyllins. Accordingly, four chlorophyllins were selected for this study.

EXPERIMENTAL

Polarography.—The capillary characteristics, the instrument used, and the procedures followed were the same as previously reported (1).

In 0.1 *M* KCl solution, a characteristic reduction wave appeared for all ten chlorophyllins. The $E^1/_2$ of this wave ranged from −1.20 to −1.38 volts *vs.* the saturated calomel electrode (SCE). The chlorophyllin not containing a metal and the cobalt chlorophyllin showed an additional wave at a slightly more positive potential.

In 1.5 *M* ammonia-ammonium chloride solution, a characteristic reduction wave appeared for 6 of the chlorophyllins. The $E^1/_2$ of this wave ranged from −0.87 to −1.02 volts *vs.* the SCE. The other four chlorophyllins were not soluble in this media and therefore could not be determined.

Five of the eight copper chlorophyllins gave two additional reduction waves at $E^1/_2$ of −0.26 and −0.53 volt *vs.* the SCE. These waves are attributed to the ionic copper present and appear even in those instances when the chlorophyllin itself is not soluble in the ammoniacal media.

Adsorption Chromatography.—In a series of exploratory chromatograms, using the method outlined by Binkley and Wolfrom (5), it was found that several components of the chlorophyllins could be separated on talc, using water or 0.1 *M* KCl solution as the developer.

A large column of talc, 28 mm. in diameter and 28 cm. in length, was prepared for each chlorophyllin. Approximately 350 mg. of the chlorophyllin was dissolved in 10 ml. of the appropriate solvent and added to the top of the column. When this solution had been adsorbed, 15-ml. portions of the solvent were added to the column daily and, by gravity flow, allowed to develop the chromatogram. Approximately 150 ml. of solvent was required for this development.

The colors of the fractions on the column ranged from a pale yellow to various shades of green. In two instances a brown coloration was observed. Solutions of the fractions, after separation from the talc by the use of appropriate solvents or elution, were spotted on Whatman filter paper and examined under ultraviolet light. Varying degrees of fluorescence were observed.

The solutions of the fractions obtained were electrolyzed at the dropping mercury electrode. In all cases, the fractions containing the reducible component could be identified polarographically.

The properties of the chromatographic fractions obtained from the various chlorophyllins are given in Table I.

DISCUSSION

As yet there is no experimental evidence as to which portion of the chlorophyllin molecule gives rise to the characteristic reduction wave, at $E^1/_2$ of −1.20 to −1.38 volts *vs.* the SCE, in 0.1 *M* KCl solution. It was believed that this wave was caused

* Received August 16, 1954 from the Research Laboratories, School of Pharmacy, Purdue University, Lafayette, Indiana.
† Present address: College of Pharmacy, Rutgers University, 1 Lincoln Avenue, Newark 4, New Jersey.

TABLE I.—PROPERTIES OF THE CHROMATOGRAPHIC FRACTIONS OF FOUR COMMERCIAL CHLOROPHYLLINS

Chlorophyllin and Concentration[a]	Polarographic Reduction[b]—$E^1/_2$ vs. SCE Fraction[c]									
	1	2	3	4	5	6	7	8	9	10
1. CS—64[d]										
133.7%				−0.94[k]	−0.92[k, l]	−0.92[k, l]	−0.90			
Company D	None[h]	−0.70[i]	−1.16[j]	−1.24	−1.24	−1.24	−1.24	−1.05[j]	−1.08[j]	−0.97[j, m]
2. Water soln.[e]										
25%										−1.38[k, m]
Company A	None	None	None	−1.50[n]	None	None	None	None	None	−1.69
3. NaKCu[f]										
78%										
Company A	−1.28	−1.30	−1.31[k]	−1.30[o]	None	None	None[r]	No other fractions obtained		
4. NaKFe[g]										
77.2%			−0.93[p, k]	−0.92[p]						
Company A	None	−0.875	−1.23	−1.26[q]	−1.50	−1.02	No other fractions obtained			

[a] Concentration figure supplied by manufacturer.
[b] Using 0.1 *M* KCl as supporting electrolyte.
[c] Fractions number from top of column.
[d] Fractions separated from talc with distilled water.
[e] Fractions separated from talc with acetone. Solutions evaporated to dryness and residue dissolved in 25 ml. of 0.1 *M* KCl.
[f] Fractions separated from talc with 0.1 *M* NaOH.
[g] Fractions separated from talc with a solution containing 55% acetone in water. Solutions evaporated to dryness and residue dissolved in 25 ml. of 0.1 *M* KCl.
[h] "None" refers to fact that no reducible component is present in the fraction.
[i] Very small wave.
[j] Drawn-out wave.
[k] Seems to be fraction containing most of the reducible component.
[l] Fractions 5 and 6 were combined because of difficulty in separating them.
[m] 25 ml. of eluant collected when eluant first showed a color.
[n] Prewave also present but $E^1/_2$ not measurable.
[o] Wave has pronounced maximum.
[p] Poorly formed third wave also present.
[q] Wave poorly formed.
[r] 50 ml. of eluant collected when eluant first showed a color.

by the aldehyde group present in the chlorophyllin *b* molecule. However, there is another structure which might cause this reduction wave. This is the carbonyl group on the isocyclic ring in the dicarboxylic chlorophyllins. There is some justification for this speculation because 4 of the chlorophyllins supplied for this investigation were known to contain the dicarboxylic form, and in these instances the increase in the height of the characteristic wave corresponds roughly with the increase in concentration of the chlorophyllin as determined by the N. N. R. method (6).

Adsorption chromatography was used in an attempt to separate the fractions responsible for the wave. Isolation of several fractions was achieved. In the polarographic determination of the various fractions obtained, it was noted that the half-wave potentials of those fractions which reacted, in most cases, were shifted to a more negative potential over that found in the original chlorophyllin determination. The presence of other fractions and impurities undoubtedly has an effect on the reduction wave of the original chlorophyllin.

The ultimate proof regarding the structure responsible for the polarographic reduction wave must wait until the synthesis of pure chlorophyllin *a* and chlorophyllin *b* is achieved. However, the preparation of a well-defined series of dicarboxylic materials and another series of tricarboxylic materials could aid materially in this determination.

REFERENCES

(1) Wuggatzer, W. L., and Christian, J. E., *Science*, **118**, 444(1953).
(2) Voigtlander, H. W., and Henning, H. M., *Arzneimittel-Forsch.*, **3**, 182(1953).
(3) Eddy, W. H., "Chlorophyll 1953," American Chlorophyll Division, Strong Cobb and Co., Inc., Lake Worth, Florida.
(4) Tiselius, A., *Endeavour*, **11**, 5(1952).
(5) Binkley, W. W., and Wolfrom, M. L., "Chromatography of Sugars and Related Substances," Scientific Report Series No. 10, Sugar Research Foundation, Inc., New York, 1948.
(6) "New and Nonofficial Remedies, 1952," J. B. Lippincott Co., Philadelphia, Pennsylvania, 1952.

Preparation and Properties of 8-Chlorotheophylline Salts of Some Alkaloids*

By M. L. ROBINETTE, Jr.† and F. W. BOPE‡

The 8-chlorotheophylline salts of five important alkaloids were prepared. Some of the more important physical properties, including optical crystallographic properties, were determined.

THE WORK OF CUSIC (1–16) involved the preparation of 8-haloxanthine salts of antihistaminic agents. The resulting compounds had reduced toxicity and enhanced therapeutic efficacy when evaluated with the corresponding antihistaminics themselves. Drowsiness, the principal untoward reaction commonly identified with antihistaminics, was neutralized in many of the cases tested.

The 8-haloxanthines such as 8-chlorotheophylline contain a hydrogen atom at position 7. These are moderately strong acidic compounds by virtue of the fact that the strongly electronegative halogen at position 8 attracts electrons from position 7, resulting in the creation of an acidic hydrogen at position 7. Therefore, it is possible to form definite salts between certain members of the haloxanthines and certain organic bases. Among the first such salts reported (3) was the 8-chlorotheophylline salt of diphenhydramine, which is commonly known as Dimenhydrinate or Dramamine.

The differences in pharmacological activity between diphenhydramine and dimenhydrinate are commonly known. On this basis, it is conceivable that the alkaloidal 8-chlorotheophyllinates may have modified pharmacological actions when compared to those of the individual alkaloids. The primary purpose of this investigation was to prepare the 8-chlorotheophylline salts of some alkaloids and to determine some of the physical properties of these salts.

EXPERIMENTAL

Optical examination by use of the polarizing microscope showed all of the alkaloidal 8-chlorotheophyllinates to be anisotropic. Useful interference figures were not found, but two refractive indices for each salt were determined by the immersion (Becke line) method (17).

The 8-Chlorotheophylline Salt of Quinine.—Approximately equimolecular quantities of 8-chlorotheophylline and quinine (2.015 Gm. and 3.123 Gm., respectively) were added to 50 ml. of 95% ethanol and 50 ml. of distilled water and refluxed for three and one-half hours. Concentration of the solvent and subsequent standing at room temperature produced white needle-shaped crystals, which were filtered out and recrystallized from distilled water. After purification by further recrystallization, the crystals were dried in the Abderhalden drying pistol over phosphorus pentoxide at reduced pressure. The melting point was 130–133°. The approximate solubility in water was 1:2,000. The total yield was 4.037 Gm. or 80%.

Optical Crystallographic Properties.—The material consisted of colorless rods. *Characters Shown in Parallel Polarized Light (Crossed Nicols).*—The crystals exhibited parallel extinction and polarizing colors. *Characters Shown in Convergent Polarized Light (Crossed Nicols).*—Partial biaxial interference figures were observed. *Refractive Indices.*—α = 1.546 (shown crosswise), β = indet., γ = 1.608 (shown lengthwise). Both ±0.002.

Anal.—Calcd. for $C_{27}H_{31}N_6O_4Cl$: C, 60.15; H, 5.79; N, 15.59; Cl, 6.57. Found: C, 60.53; H, 5.78; N, 15.53; Cl, 6.37.

The 8-Chlorotheophylline Salt of Ephedrine.—Approximately equimolecular quantities of 8-chlorotheophylline and ephedrine (5.012 Gm. and 3.858 Gm., respectively) were refluxed with 50 ml. of 95% ethanol for two hours. The resulting solution was cooled to room temperature and allowed to stand overnight. When no crystallization occurred, the solution was concentrated to approximately one-half its volume and placed in the refrigerator for forty-eight hours. The resulting crystals were filtered and recrystallized twice from 95% ethanol. Finally the crystals were filtered, washed with several small portions of diethyl ether, allowed to air-dry and then dried at 65° for three hours over phosphorus pentoxide in the Abderhalden drying pistol at reduced pressure. The melting point was 86–89°. The approximate solubility in water was 1:250. The total yield was 5.602 Gm. (63%).

Optical Crystallographic Properties.—The material consisted of elongated needle-shaped colorless crystals. *Characters Shown in Parallel Polarized Light (Crossed Nicols).*—The crystals exhibited parallel extinction and showed little polarizing colors. *Characters Shown in Convergent Polarized Light (Crossed Nicols).*—Partial biaxial interference figures were observed. *Refractive Indices.*—α = 1.471 (shown crosswise), β = indet., γ = 1.614 (shown lengthwise). Both ±0.002.

Anal.—Calcd. for $C_{17}H_{23}N_6O_4Cl$: C, 53.75; H, 5.83; N, 18.44; Cl, 9.33. Found: C, 54.29; H, 5.74; N, 18.32; Cl, 9.14.

The 8-Chlorotheophylline Salt of Morphine.—Approximately equimolecular quantities of 8-

* Received August 11, 1954 from the College of Pharmacy, Ohio State University. Based in part upon a thesis submitted by M. L. Robinette, Jr. to the Graduate School of The Ohio State University in partial fulfillment for the degree of Master of Science.

† Present address: R.F.D. 2, Box 107, Ashland, Ky.
‡ Assoc. Prof. of Pharmacy, College of Pharmacy, The Ohio State University, Columbus.

chlorotheophylline and morphine (1.412 Gm. and 2.013 Gm., respectively) were added to 100 ml. of 95% ethanol and the mixture was refluxed gently for three hours. The resulting solution was then concentrated to approximately one-half its volume and allowed to stand at room temperature. After twenty-four hours, a fine precipitate formed, which was removed by filtration and recrystallized twice from 95% ethanol. Finally, the product was dried in the manner described above. The product sublimed at 223°. The approximate solubility in water was 1:6000. The yield was 1.521 Gm. (33%).

Optical Crystallographic Properties.—The material occurred as elongated needle-shaped crystals. *Characters Shown in Parallel Polarized Light (Crossed Nicols).*—The crystals exhibited parallel extinction and had little polarizing color. *Characters Shown in Convergent Polarized Light (Crossed Nicols).*—Partial biaxial interference figures were demonstrated. *Refractive Indices.*—α = 1.544 (shown lengthwise), β = indet., γ = 1.550 (shown crosswise). Both ± 0.002.

Anal.—Calcd. for $C_{24}H_{28}N_8O_4Cl$: C, 57.65; H, 5.24; N, 14.01; Cl, 7.09. Found: C, 57.95; H, 5.31; N, 13.99; Cl, 6.77.

The 8-Chlorotheophylline Salt of Strychnine.—Approximately equimolecular quantities of 8-chlorotheophylline and strychnine (1.135 Gm. and 2.003 Gm., respectively) were dissolved in 200 ml. of chloroform with the aid of heat, and the mixture was refluxed for one hour. The solvent was then allowed to evaporate spontaneously to approximately one-half its volume. The resulting crystals were recrystallized twice from 95% ethanol and dried in the manner described above. The crystals decomposed above 264°. The approximate solubility in water was 1:2,500. Yield, 0.614 Gm. (21%).

Optical Crystallographic Properties.—The material appeared as rod-shaped crystals. *Characters Shown in Parallel Polarized Light (Crossed Nicols).*—The crystals exhibited parallel extinction and had brilliant polarizing colors. *Characters Shown in Convergent Polarized Light (Crossed Nicols).*—Partial biaxial interference figures were demonstrated. *Refractive Indices.*—α = 1.549 (shown crosswise), β = indet., γ = 1.561 (shown lengthwise). Both ±0.002.

Anal.—Calcd. for $C_{28}H_{32}N_8O_4Cl$: C, 61.25; H, 5.32; N, 15.31; Cl, 6.45. Found: C, 62.63; H, 5.48; N, 14.82; Cl, 5.17.

The 8-Chlorotheophylline Salt of Brucine.—Approximately equimolecular quantities of 8-chlorotheophylline and brucine (1.103 Gm. and 1.981 Gm., respectively) were refluxed gently for one hour with 100 ml. of 95% ethanol. The solution was concentrated to approximately one-half its volume, and the resulting crystals were recrystallized twice from 95% ethanol and dried in the manner described above. The melting point was 282–285°. The approximate solubility in water was 1:2,500. The total yield was 1.325 Gm. or 42%.

Optical Crystallographic Properties.—The material appeared as colorless rods. *Characters Shown in Parallel Polarized Light (Crossed Nicols).*—The crystals exhibited parallel extinction. *Characters Shown in Convergent Polarized Light (Crossed Nicols).*—Partial biaxil interference figures were observed. *Refractive Indices.*—α = 1.473 (shown lengthwise), β = indet., γ = 1.737 (shown crosswise). Both ±0.002.

Anal.—Calcd. for $C_{50}H_{54}N_{16}O_8Cl$: C, 59.15; H, 5.46; N, 13.79; Cl, 5.82. Found: C, 62.99; H, 5.89; N, 13.62; Cl, 0.56. The composition of the 8-chlorotheophylline salt of brucine was in doubt.

SUMMARY

Attempts were made to prepare the 8-chlorotheophylline salts of five alkaloids, namely brucine, ephedrine, morphine, quinine, and strychnine. The respective melting points and optical crystallographic properties were determined, as well as their approximate solubilities in water.

It was possible to form 8-chlorotheophylline salts of the alkaloids ephedrine, morphine, quinine, and strychnine, but the reaction of 8-chlorotheophylline with brucine produced a compound of doubtful composition as indicated by the analytical data. Each salt which was prepared exhibited a melting point which was different from the melting point of the original alkaloid, of 8-chlorotheophylline, or of a mechanical mixture of the alkaloid and 8-chlorotheophylline. The salts were less soluble in water than the corresponding free alkaloidal bases. The salts exhibited characteristic optical crystallographic properties. All of those prepared were found to be anisotropic.

REFERENCES

(1) Cusic, J. W., U. S. pat. 2,499,058.
(2) Cusic, J. W., *Science*, **109**, 574(1949).
(3) Cusic, J. W., U. S. pat. 2,534,235.
(4) Cusic, J. W., U. S. pat. 2,534,236.
(5) Cusic, J. W., U. S. pat. 2,534,237.
(6) Cusic, J. W., U. S. pat. 2,534,238.
(7) Cusic, J. W., U. S. pat. 2,534,239.
(8) Cusic, J. W., U. S. pat. 2,534,240.
(9) Cusic, J. W., U. S. pat. 2,534,241.
(10) Cusic, J. W., U. S. pat. 2,534,242.
(11) Cusic, J. W., U. S. pat. 2,534,243.
(12) Cusic, J. W., U. S. pat. 2,534,244.
(13) Cusic, J. W., U. S. pat. 2,534,245.
(14) Cusic, J. W., U. S. pat. 2,534,246.
(15) Cusic, J. W., U. S. pat. 2,534,247.
(16) Cusic, J. W., U. S. pat. 2,534,813.
(17) Chamot, E. M., and Mason, C. W., "Handbook of Chemical Microscopy," 2nd Ed., John Wiley & Sons, Inc., New York, 1951.

Studies in the Stabilization of Folic Acid in Liquid Pharmaceutical Preparations*

By ROBERT P. TANSEY† and GEORGE H. SCHNELLER‡

Of a number of compounds classified as antioxidants and known to have some stabilizing effect in other pharmaceutical preparations, it was found that nordihydroguaiaretic acid, butylated hydroxyanisole, and ethyl hydrocaffeate substantially improved the stability of folic acid in certain types of acqueous and simple solutions containing folic acid in the presence of riboflavin. Concentrations of 0.02 to 0.05 per cent of these compounds were found to stabilize folic acid both in the presence and absence of light.

THE BROAD usefulness of folic acid in therapeutics and in nutrition makes desirable its inclusion in a variety of pharmaceutical preparations. In solid products such as tablets and capsules, folic acid usually presents no serious difficulty in the way of stability upon prolonged storage. However, in certain types of aqueous or hydroalcoholic products the stability of folic acid has presented a practical problem. Scheindlin (1) showed that folic acid in the form of an elixir at *p*H 8.2 without any other therapeutic agents present was completely stable over a four-month period of storage at room temperature. Biamonte and Schneller showed the stability of folic acid in aqueous solution (2) to be in part a function of the *p*H, the stability being lower at lower *p*H values. Scheindlin and Griffith (3) showed that ascorbic acid has a deleterious effect upon folic acid in solution, and the mechanism of the reaction was studied at some length.

Considerable research has been done on the influence of various members of the vitamin B complex upon folic acid stability in solution. Biamonte and Schneller (2) found that riboflavin had a very marked destructive effect on folic acid in solution, while thiamine hydrochloride had a less serious effect. They found niacinamide, pyridoxine, and pantothenyl alcohol relatively innocuous. In the case of riboflavin, they showed that the decomposition of folic acid involves the liberation of free *p*-aminobenzoylglutamic acid, indicating a cleavage at the methylene bridge.

Scheindlin, Lee, and Griffith (4) studied the reaction between riboflavin and folic acid in considerable detail and made the following observations:

(*a*) The reaction is greatly accelerated by light. (*b*) the reaction is not bimolecular, but the riboflavin causes the breakdown of folic acid even when present in a molar ratio of 1 to 166. Also, when riboflavin is present in an equimolar ratio, it can be recovered essentially unreduced even after the complete cleavage of the folic acid. (*c*) The reaction is retarded, but not halted, by the exclusion of air. Those workers proposed the following possible mechanism for the reaction. The folic acid is oxidized by a molecule of riboflavin in the presence of light, the ultimate oxidation products being *p*-aminobenzoylglutamic acid and 2-amino-4-hydroxy-6-pteridine-carboxaldehyde. In the course of its participation in the oxidation of folic acid, the riboflavin is reduced to leucoriboflavin, which in the presence of atmospheric oxygen is reoxidized to riboflavin which can re-enter the cycle.

In view of this evidence of oxidative nature of the reaction involved in the decomposition of folic acid in the presence of riboflavin, it was considered reasonable to test various known antioxidants for possible stabilizing action. The results of such tests comprise the body of this report.

EXPERIMENTAL

Aqueous solutions containing folic acid and riboflavin were tested for folic acid stability upon (*a*) short-term exposure to direct daylight, (*b*) storage in a closed chamber for twenty-one days at 45°, and (*c*) storage for variable periods in subdued light at room temperature.

In these experiments the simplest possible vehicle was used. B-vitamin preparations are usually adjusted to rather low *p*H levels to enhance the stability of other factors than folic acid. Therefore, most of the samples prepared were in the range of *p*H 4.5 to 5.5. A few tests were carried out at higher *p*H

Presented at the Annual Meeting of the Scientific Section of the AMERICAN PHARMACEUTICAL ASSOCIATION, Salt Lake City, August 19, 1953.

* Received August 2, 1953 from the Calco Chemical Division, American Cyanamid Company, Bound Brook, N. J.

† Present address Schering Corporation, 86 Orange St., Bloomfield, N. J.

‡ Present address American Cyanamid Company, 30 Rockefeller Plaza, New York 20, N. Y.

Mr. Edmund J. Gulick performed most of the analytical work involved in this study.

levels. Typically, the composition of the test solution was as follows:

	Gm. per 100 cc.
Folic Acid	0.01
Riboflavin	0.01
Propylene Glycol	(Variable)
Methylparaben	0.05
Propylparaben	0.02
Citric Acid-Disodium Acid Phosphate Buffer	*q. s.*

The purpose of the propylene glycol was to aid in solubilizing the folic acid (2) as well as the parabens and the stabilizers. The parabens were used to assure mold-free preparations during storage periods. In one series lactic acid was used in place of citric acid in the buffer system, with no marked difference in the results. The amount of buffer was sufficient to maintain the desired *p*H level throughout the study. In some of the solutions niacinamide was used as an auxiliary solubilizing agent for the folic acid (5, 6).

The folic acid and the riboflavin were used in equal concentrations because their customary dosage is of the same order of magnitude. The influence of variation in the riboflavin concentration upon the rate of decomposition of the folic acid was not tested because Scheindlin (4) had shown that riboflavin catalyzes the decomposition even when present in minute relative concentrations, and that the rate of decomposition is high when the riboflavin concentration approximates that of the folic acid.

The solutions were assayed immediately after preparation and again after storage under various conditions. One sample was kept in an incubator at 45° for twenty-one days. Other samples were stored at room temperature in subdued light (i. e., in cartons holding 12 to 24 bottles and open at the top, stored on shelves remote from windows). The preceding two types of sample were stored in amber glass screw-capped bottles. Since folic acid had been shown by Stokstad and his co-workers to be subject to photolysis when exposed to direct sunlight (7) and since Scheindlin had shown that the degradation under study is markedly accelerated by exposure to daylight (4), a sample of each test solution was also exposed to direct daylight in a flint glass screw-capped bottle. The purpose of this was to determine whether such samples could be used in an accelerated test to predict what the stability would be under less rigorous storage conditions.

A number of compounds classified as antioxidants and known to have some stabilizing effect in other pharmaceutical systems were tested. Among these were nor-dihydroguaiaretic acid,[1] butylated *p*-hydroxyanisole,[2] and ethyl hydrocaffeate.[3] Other antioxidants which proved less active in our tests are listed later in this report.

ANALYTICAL PROCEDURE

The extent of folic acid decomposition in the test samples was determined by a procedure based upon that developed by Kaselis and co-workers (8). In this procedure, free *p*-aminobenzoylglutamic acid (PABG), which is the decomposition product of folic acid, is determined directly by diazotization and coupling with the Bratton and Marshall reagent (9). Colorimetric estimation and the use of a calibration curve give the amount of decomposed folic acid (PABG) present in the sample. Total folic acid (both decomposed and intact) in the sample can be determined by another step in the procedure, involving treatment with zinc amalgam, whereby the folic acid is reduced to PABG. The latter is estimated as before. In practice, the "total folic acid" assay value usually remained at the level present initially, even where the free PABG level rose to quite high levels, for example, levels equivalent to 50% decomposition of the folic acid. This showed that the PABG formed by the decomposition underwent no further change involving the amine group. It was feasible, therefore, to shorten the time spent in analysis by omitting the "total folic acid" determination and relying solely on the free PABG value, after the initial assay on the sample had indicated the proper content of total folic acid. However, when the degree of breakdown was excessive, such as above 80%, the "total folic acid" value was found to be considerably lower than originally, indicating further breakdown of the PABG by some unknown mechanism. An example of this is shown in Table I. The samples concerned are the same as those dealt with in Table II.

TABLE I.—PABG AND "TOTAL FOLIC ACID" ASSAYS OF FOLIC ACID-RIBOFLAVIN SOLUTIONS ON AGING

Storage Conditions	PABG[a] (as Folic Acid), mg. per 25 cc.	Total Folic Acid,[b] mg. per 25 cc.	Decomposition,[c] %
Without Antioxidant			
Initial	0.46	2.30	20
21 days, 45°	0.88	2.24	40
1 month, RT[d]	1.36	2.31	59
20 days, direct light	1.25	1.20	100
With NDGA 0.02%			
Initial	0.10	2.34	4.3
21 days, 45°	0.35	2.30	15
1 month, RT	0.16	2.43	7
20 days, direct light	1.96	2.44	80

[a] PABG resulting from decomposition of folic acid.
[b] Includes both intact and decomposed folic acid (PABG).
[c] PABG/total folic acid.
[d] RT—room temperature.

RESULTS

Using the test solution described above, containing 25% V/V of propylene glycol and 5% of niacinamide, the three antioxidants, nordihydroguaiaretic acid (NDGA), butylated hydroxyanisole (BHA), and ethyl hydrocaffeate (EHC), had protective activity when included in reasonable concentrations. This is shown in Table II. Similar results were obtained when the base contained only 15% of propylene glycol but in addition 20% of sucrose.

[1] Obtained from William J. Stange Company, Chicago.
[2] A mixture of 2-tert.butyl-4-hydroxyanisole and 3-tert.butyl-4-hydroxyanisole, obtained from Universal Oil Products Company.
[3] Ethyl ester of 3,4-dihydroxyphenyl propionic acid, obtained from R. W. Greeff & Co., New York, N. Y.

TABLE II.—PROTECTIVE EFFECT OF ANTIOXIDANTS ON FOLIC ACID-RIBOFLAVIN MIXTURES AT *p*H 4.5

Antioxidant	Decomposition of Folic Acid, %					
	Initial	5 days/light[a]	21 days/45°	1 mo./RT[b,c]	3 mo./RT[b]	6 mo./RT[b]
None	20	100	40	59	..	65
NDGA, 0.02%	4.3	53	15	7	10	16
BHA, 0.05%	4.5	20	14	9	11	17
EHC, 0.05%	1.6	16	13	7	10	11

[a] Direct daylight in flint glass bottles.
[b] Diffused daylight in amber glass bottles.
[c] RT—Room temperature.

At *p*H 5.5 in the same system, the protective influence of these compounds was less marked, though still substantial, as shown in Table III. At higher *p*H levels the protective effect was still less pronounced.

In similar experiments chemically equivalent amounts of riboflavin-5′-phosphate ester monosodium salt dihydrate or riboflavin-5′-phosphate ester mono-diethanolamine salt dihydrate were used in place of U.S.P. riboflavin. The antioxidants exerted a strong protective effect here also.

Propyl gallate had a lesser but significant protective effect. Melilotin (dihydrobenzopyrone) had only a slight effect. The following compounds had an insignificant degree of protective power, since solutions at *p*H 4.5 containing them in the indicated concentrations decomposed to the extent of 60% or more during six month's storage at room temperature:

Sodium formaldehyde sulfoxylate, %	0.02
Sodium bisulfite, %	0.10
Thioglycerol, %	0.10
Thiourea, %	0.02
Glycine, %	1.00
Ascorbyl palmitate, %	0.05

A series of samples was prepared in a medium similar to that in the basic series described above, but containing in addition to 25% V/V of propylene glycol, 16% of sucrose and 0.15 Gm. of sodium carboxymethylcellulose (high-viscosity type) as a suspending agent. This series contained per 100 cc.: folic acid and riboflavin, each 0.015 Gm., nicotinamide 0.2 Gm., thiamine hydrochloride 0.02 Gm., and pyridoxine 0.01 Gm. The *p*H was adjusted to 4.5. In this series the folic acid was not in complete solution, which accounts for the exceptionally favorable stability observed (2). The results are shown in Table IV.

TABLE III.—PROTECTIVE EFFECT OF ANTIOXIDANTS ON FOLIC ACID-RIBOFLAVIN MIXTURES AT *p*H 5.5

Antioxidant	Decomposition of Folic Acid, %					
	Initial	5 days/light[a]	21 days/45°	1 mo./RT[b,c]	3 mo./RT[b]	6 mo./RT[b]
None	19	100	43	56	..	83
NDGA, 0.02%	3.5	11	18	9	18	35
BHA, 0.05%	3.9	8	14	9	17	34
EHC, 0.05%	2.4	6	13	7	17	33

[a] Direct daylight in flint glass bottles.
[b] Diffused daylight in amber glass bottles.
[c] RT—room temperature.

TABLE IV.—PROTECTIVE EFFECT OF ANTIOXIDANTS ON FOLIC ACID IN B-COMPLEX VITAMIN MIXTURES

Antioxidant	Decomposition of Folic Acid, %					
	Initial	10 days/light[a]	21 days/45°	1 mo./RT[b,c]	3 mo./RT[b]	6 mo./RT[b]
None	7.7	95	36	14	15	11
NDGA, 0.02%	3.6	4.3	18	4.6	5.4	10
BHA, 0.05%	4.3	5.7	23	6.0	9.0	13
EHC, 0.05%	3.7	4.3	22	4.0	4.0	8.0
NDGA, BHA, EHC[d]	4.6	5.6	14	4.6	6.0	10

[a] Direct daylight in flint glass bottles.
[b] Diffused daylight in amber glass bottles.
[c] RT—room temperature.
[d] NDGA 0.02% + BHA 0.05% + EHC 0.05%.

TABLE V.—PROTECTIVE EFFECT OF ANTIOXIDANTS ON A FOLIC ACID-RIBOFLAVIN MIXTURE CONTAINING 15% NIACINAMIDE

Antioxidant	Decomposition of Folic Acid, %						
	Initial	10 days light[a] Amber	10 days light[a] Flint	21 days/45°	1 mo./RT[b,c]	3mo./RT[b]	6 mo./RT[b]
None	33	60	..	55	25	59	100
NDGA, 0.02%	5	26	100	19	11	14	35
BHA, 0.05%	5	8	100	15	4	14	26
EHC, 0.05%	4	10	84	18	5	16	28

[a] Samples stored in amber and flint glass bottles.
[b] Amber glass bottles.
[c] RT—room temperature.

Another series of samples contained no propylene glycol or sucrose but only niacinamide (15 Gm. per 100 cc.) as a solubilizer for the folic acid, and ethanol (1%) to dissolve the antioxidants. This series contained no parabens. The *p*H was adjusted to 4.5. The results are shown in Table V.

DISCUSSION

The experimental results reported herein confirm the report of Scheindlin, Lee, and Griffith (4) to the effect that the reaction between folic acid and riboflavin is accelerated by light and that the reaction proceeds when the solution is kept in amber glass containers. The effectiveness of these antioxidants in retarding the rate of decomposition of folic acid in the presence of riboflavin appears to lend support to the oxidative reaction mechanism postulated by them. However, this concept is not to be considered as having been proved, as far as the breakdown of folic acid under normal storage conditions is concerned. The reduced rate of decomposition taking place in these simple solutions during storage periods of six months gives rise to the hope that the use of such antioxidants may prove of some practical aid in the compounding of liquid preparations having a more satisfactory folic acid stability. Preliminary studies indicate that this hope may be realized with certain types of products. Further studies are under way.

It will be noted that the initial assay of all the unstabilized control samples indicates gross decomposition. It should be explained that the initial assay was carried out after a variable time interval following the completion of the preparation of the sample, with a limit of approximately twenty-four hours. Scheindlin (4) reported that solutions of this type decomposed to the extent of as much as 22.7% in one day. In the course of these experiments, no extreme precautions were taken to reduce to a minimum the amount of decomposition taking place during the preparation of the solutions and up to the time of the initial assay. The distilled water used was recently boiled to reduce the oxygen content at the time of use, and the samples were prepared in an area of subdued light. However, the combined effect of saturating the water and the finished solution with nitrogen, of preparing the solution in a photographic darkroom and keeping the sample in a completely opaque container, and of assaying the sample immediately after preparation, was not tested. It is reasonable to expect that under such strict control, the initial assay would indicate a lower degree of decomposition, even in samples containing no antioxidant.

The correlation between the samples exposed to direct light in flint glass and those stored in subdued light in amber glass is rather poor. A comparison between Tables II and III shows that in one series (Table II), a relatively high degree of decomposition in the direct daylight test was associated with relatively good stability during six months' storage in subdued light. In another (Table III) the reverse is true. Furthermore, the NDGA sample in Table II appears to have undergone an excessive amount of decomposition in direct light compared to what would be expected on the basis of the results in subdued light. These anomalies may be explained in considerable part by the variability of daylight as a source of illumination. The development of an "accelerated storage" test might be feasible with an appropriate source of artificial illumination. To establish the validity of such a test, it would also be necessary to control the exposure to illumination of the long-range samples more strictly than was done in this study. On the other hand, it must be remembered that the reaction takes place in the dark, as reported by Scheindlin (4) and as shown in the above data obtained with samples stored in amber glass bottles in an incubator with a dark interior. It is quite possible that the light-catalyzed reaction and the slower one taking place in the dark proceed by two entirely different mechanisms. Since most vitamin preparations are packaged in amber glass with an exterior cardboard container, the product is exposed to very little light. Therefore, the long-range observations shown in the tables are no doubt of much greater significance for practical purposes than the results of the short range light-accelerated tests.

SUMMARY

1. Certain phenolic antioxidants were found capable of retarding to a substantial degree the light-catalyzed cleavage of folic acid occurring in the presence of riboflavin.

2. The same antioxidants also had a demonstrable protective effect upon simple folic acid-riboflavin solutions kept in the dark.

3. In relatively simple solutions, the antioxidants found most effective were nordihydroguaiaretic acid, butylated hydroxyanisole, and ethyl hydrocaffeate. They retarded the reaction significantly when present in concentrations of 0.02 to 0.05%.

REFERENCES

(1) Scheindlin, S., *Am. J. Pharm.*, **120**, 103(1948).
(2) Biamonte, A. R., and Schneller, G. H., THIS JOURNAL, **40**, 313(1951).
(3) Scheindlin, S., and Griffith, I., *Am. J. Pharm.*, **123**, 78(1951).
(4) Scheindlin, S., Lee, A., and Griffith, I., THIS JOURNAL, **41**, 420(1952).
(5) Taub, A., and Lieberman, H., *ibid.*, **42**, 183(1953).
(6) Biamonte, A. R., and Schneller, G. H., "Calco Applications," American Cyanamid Company, No. 64, May, 1953.
(7) Stokstad, E., Fordham, D., and Degrunigen, A. D., *J. Biol. Chem.*, **167**, 877(1947).
(8) Kaselis, R. A., Leibmann, W., Seaman, W., Sickels, J. P., Stearns, E. I., and Woods, J. T., *Anal. Chem.*, **23**, 746 (1951).
(9) Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, **128**, 537(1939).

The Saponins of Pachyrrhizus Erosus (L) Urban*

By RALPH F. SHANGRAW† and E. V. LYNN‡

The seeds of *Pachyrrhizus erosus* (L) Urban are known to contain at least two saponins. These compounds were separated by extraction with methanol and found to be amorphous. They were toxic to goldfish in a concentration of 1:10,000, about 40 per cent of the potency of picrotoxin. The seeds were also toxic to goldfish in a concentration of 1:500. Two crystalline sapogenins were isolated after hydrolysis and were analyzed. They are apparently different from any sapogenins previously reported.

THE TAXONOMY of *Pachyrrhizus erosus* (L) Urban (probably *P. angulatus* Rich.) has been studied exhaustively by Clausen (1), and a study of the morphology of the seed has been made by Baker and Quimby (2).

The seeds have received attention from many chemists during the last seventy years, chiefly because the natives in tropical countries have used them as a source of fish poison. Several compounds, including rotenone and pachyrrhizid, have been separated and named, but it is doubtful if any of these compounds is in sufficient amount or potent enough to account for the poisonous character of the seed. Baker and Lynn (3), who recently gave a complete review of the literature since the first examination in 1880, established the presence of at least two water-soluble saponins. In the present investigation, the writers have attempted to isolate and study these saponins.

EXPERIMENTAL

As has been found previously, great difficulty was experienced in preparing an aqueous extract. The coarsely ground seed was exhausted with ether to remove fat, and the residue was extracted in a Soxhlet apparatus with distilled water. After a few hours the residual matter in the thimble massed in such a manner as to prevent further extraction. Maceration of ground, fat-free seeds with water in a beaker gave a gelatinous extract that could not be filtered readily, even after a preliminary straining. A satisfactory solution was prepared finally by straining through muslin, boiling the liquid to coagulate the mucilaginous matter, and filtering while hot. The clear filtrate foamed on shaking, and laked blood even after dilution.

The filtrate gave with 10% solution of lead acetate a precipitate that was yellow in color. This precipitate was suspended in water, and the mixture was treated with sodium carbonate to remove the lead, and filtered. The clear, aqueous liquid laked blood and upon evaporation left a brownish residue, which was found to be soluble in hot ethanol or in hot methanol.

The filtrate from lead acetate gave, with a solution of lead subacetate, a copious yellow precipitate, which was freed of lead in the same manner as before. The lead-free filtrate laked blood and, upon evaporation, left a light brown residue which was found to be soluble in hot ethanol or in hot methanol.

In view of the solubilities thus disclosed, the method of extraction was changed. The coarsely ground, fat-free seeds were heated with about two volumes of methanol under a reflux condenser for about one hour, and the hot mixture was filtered. After cooling overnight, the crude saponins separated in the form of a light yellow, fluffy precipitate. A similar substance was obtained by extracting the fat-free seeds with hot ethanol. By evaporation of the mother liquid in each case to half its original volume, a further quantity of insoluble material was obtained. The residual marc was repeatedly extracted with hot methanol, and more crude substance was obtained by precipitating the evaporated mother liquids with ether. A total of about 1% was collected finally.

The crude saponins appeared to be crystalline when first separated, but after washing and drying formed a yellowish, gummy mass. Attempts to recrystallize the mass using various solvents failed. From a study of the melting points of the various fractions, it appeared that at least two compounds were present, one melting at 68 to 85°, the other at more than 180°. An aqueous solution of the crude mixture laked blood, gave only a slight reduction of Fehling's solution, and foamed on shaking.

The crude saponins and also the seeds were found to be definitely toxic to fish. The saponin mixture was dissolved in water in varying concentrations, and goldfish weighing 1.0–1.5 Gm. were immersed in the solution. At a concentration of 1:10,000 the fish died within fifteen to twenty minutes after periods of excitement, motor weakness, and paralysis. In comparative tests it was found that picrotoxin killed the fish after similar symptoms in a concentration of 1:25,000. An aqueous extract of the ground seeds also killed goldfish under similar circumstances; the concentration required was about 1:500, although lower amounts stunned the fish and rendered them inactive.

The mixed saponins were dissolved in a 50% aqueous methanol containing 5% HCl and hydrolyzed by boiling under a reflux condenser. A small

* Received June 7, 1954, from the Samuel M. Beat Research Laboratory, Massachusetts College of Pharmacy.
Presented to Subsection Np, American Association for the Advancement of Science, Boston meeting, December, 1953.

† Abstracted from a thesis submitted to the Graduate Council of the Massachusetts College of Pharmacy by Ralph F. Shangraw in partial fulfillment of the requirements for the degree of Master in Science in Pharmacy.

‡ Research Professor of Chemistry, Massachusetts College of Pharmacy.

amount of crystalline matter began to appear on the sides of the flask after only one hour. At the end of twenty hours, the mixture was evaporated to dryness, leaving a dark brown mass containing the sapogenins. After experimenting with various solvents, this mass was extracted in a Soxhlet apparatus with carbon tetrachloride, and removal of the solvent left a light olive-colored residue.

Hot methanol removed about half from this mixture and upon refrigeration deposited yellowish crystals (A) which were long, narrow, and branched. The product A was treated with charcoal and recrystallized repeatedly from hot methanol and from isopropyl alcohol. The final product, m. p. 196–198°, was nicely crystalline, slightly colored, and gave a positive Liebermann-Burchard reaction.

Anal.—Calcd. for $C_{30}H_{48}O_6$: C, 72.85; H, 7.74; mol. wt., 494.6. Found: C, 72.81; H, 7.69; mol. wt. (Rast), 489.9.

The residue from A was extracted with hot isopropyl alcohol to produce a compound B. This compound, after being treated with charcoal and recrystallized, formed clusters of needle-shaped crystals with a faintly yellow color and a melting point of 176°. They gave a positive reaction in the Liebermann-Burchard test.

Anal.—Calcd. for $C_{27}H_{18}O_7$: C, 68.00; H, 4.45; mol. wt., 406. Found: C, 68.15; H, 4.50; mol. wt. (Rast), 392.

The sapogenins were submitted to the method of Walens, *et al.* (4), in order to compare them with other compounds of the class. Exactly 5 mg. of each was placed in separate 10-ml. volumetric flasks, 94% sulfuric acid was added to the mark, and the flasks were immersed in a water bath at 40° for sixteen hours. After cooling to room temperature, the content of each flask was diluted with 94% sulfuric acid to a concentration of 10 μg per milliliter. The diluted solutions were examined in a Beckman DU spectrophotometer over the range of 220–600 mμ, using 94% sulfuric acid as the blank. Sapogenin A had maxima at 240, 320, and 525; sapogenin B at 237, 275, 318, and 530. These figures did not correspond with the ones given for any of the sapogenins noted by Walens, *et al.*, although a sample of cholesterol used for comparison gave the figures reported by them.

The saponins of the seed seem to be different from any previously recorded. Therefore, we suggest the names Pachysaponin A and Pachysaponin B for the saponins and Pachysapogenin A and Pachysapogenin B for the hydrolytic products, respectively.

SUMMARY

1. The seeds of *Pachyrrhizus erosus* (L) Urban were found to contain water-soluble, amorphous Pachysaponin A and Pachysaponin B. As hydrolytic products there were isolated crystalline Pachysapogenin A, $C_{30}H_{48}O_6$, melting at 196–198°, and crystalline Pachysapogenin B, $C_{27}H_{18}O_7$, melting at 176°.

2. The crude saponin mixture was found to be fatal to goldfish in a concentration of 1:10,000. Using an aqueous extract, the seeds were fatal to similar goldfish in a concentration of 1:500.

REFERENCES

(1) Clausen, R. T., *N. Y. Cornell Univ., Agr. Expt. Sta. Mem.*, **1944**, 264.
(2) Baker, B. Y., and Quimby, M. W., THIS JOURNAL **42**, 471(1953).
(3) Baker, B. Y., and Lynn, E. V., *ibid.*, **42**, 117(1953).
(4) Walens, H. A., Turner, A., and Wall, M. E., *Anal. Chem.*, **26**, 325(1954).

A Pharmacognostical Study of Pyrularia Pubera Michx.*

By EDWARD P. CLAUS†

The taxonomy, distribution, and habitat of *Pyrularia pubera* are recorded. A study of the chief morphological and histological features of the immature and mature fruits and seeds is described.

A REVIEW of the scientific literature pertaining to *Pyrularia pubera* Michaux has disclosed that the pharmacognostical characteristics of this plant have not been reported. In 1933 a publication on the therapeutic action of fluidextracts prepared from the fruit and from the seed (1) offered brief morphological information. Recently a phytochemical study of the pericarp (2) and the analytical constants of the fixed oil derived from the seed (3) have been determined. Currently, a pharmacological investigation of the seeds is being prepared for publication (4). In view of the interest in this plant, it was deemed advisable to record the taxonomy, distribution, and habitat of *Pyrularia pubera* and to describe the morphological characteristics and the chief histological features of the fruit and seed as well as other organs of the plant.

TAXONOMY

Pyrularia pubera Michx. is a member of the *Santalaceae* (Sandalwood Family). This family is composed largely of tropical parasitic and semiparasitic forms and is classified adjoining the

* Presented to the Scientific Section, A. PH. A., Boston meeting, August 26, 1954.
† Professor of Pharmacognosy, University of Pittsburgh, School of Pharmacy, Pittsburgh, Pa.

Loranthaceae (Mistletoe Family), also noted for its parasitic species. In 1803 the French botanist, Michaux, originated the genus name inasmuch as the term *Pyularia* is the diminutive form of *Pyrus* (pear) which the fruit resembles in appearance. According to "Index Kewensis" (5) other early botanists also classified the plant and ascribed various names to it: *Hamiltonia* by Muhlenberg in 1806, *Calinux* by Rafinesque in 1808, and *Sphaerocarya* by Wallich in 1824. Following "International Rules of Botanical Nomenclature" (6) the name *Pyularia* is now the accepted genus. Although "Gray's Manual of Botany" (7) applied the synonyms of Buffalo-nut and Oil-nut to the genus *Pyularia*, these two synonyms were listed for the species, *P. pubera*, by "The New Britton and Brown Illustrated Flora" (8). Only the name Buffalo-nut was given for the genus in "Plant Names, Scientific and Popular" (9); however, the name oil-nut, and in addition the name elk-nut, signified the species.

Evidently the oil-bearing properties of this plant have been recognized for many years judging by the specific name *oleifera* which was employed by early investigators: *Pyularia oleifera* A. Gray and *Hamiltonia oleifera* Muhlenberg. The 1933 publication previously referred to (1) used the Asa Gray nomenclature, *Pyularia oleifera*. The same article lists two additional common names: crazy nut and mother-in-law nut. These apparently are only local names since they cannot be located in other books or publications. One additional synonym might be mentioned: Clute (10) applied the term rabbit-wood to the species, *P. pubera*.

DISTRIBUTION AND HABITAT

The genus *Pyularia* consists of three species: two are Asiatic trees, the third is a shrub growing in North America. Based upon a survey of authenticated herbarium specimens, Demoise (11) determined the geographical distribution of the American species. From its northernmost stations in Westmoreland and Fayette counties in western Pennsylvania, *Pyularia pubera* extends through the mountainous districts of West Virginia, Kentucky, Virginia, North Carolina, Tennessee, South Carolina, and Alabama, reaching its southernmost limit on Stone Mountain east of Atlanta, Georgia. This range suggests that it once formed a part of the cretaceous flora of North America and that its present distribution is the result of past glacial migrations. Within the past few years, newly discovered stations have extended its northward range in Pennsylvania possibly indicative of the return of the plant to its original habitat.

The plant is described (7, 8) as parasitic on the roots of woody plants; nevertheless it possesses the normal green appearance of physiologically independent plants. Its leaves are retained longer than most other deciduous plants. Demoise (11) stated it to be a partial parasite on various species of *Calycanthus*, *Kalmia*, *Quercus*, *Castanea*, and *Tsuga*. Preferring the slopes of hills, the shrub may be found in rich soil in partially shaded woodlands of the Appalachian region. Many of the young plants have been observed as offshoots from the elongated root systems of larger neighboring plants.

MORPHOLOGY

The following morphological descriptions are based on field observations and collections made at several stations of the plant, particularly in Fayette County at Ohiopyle, Pennsylvania. Flowers, leaves, and developing fruits were gathered at intervals during the 1954 growing season in order to study the changes in external appearance as well as in internal structure. At the time of reporting this study, the fruits had reached maturity but had not ripened; thus, certain observations may be incomplete. The fresh material was either examined immediately, refrigerated and studied within forty-eight hours, or preserved in 70% alcohol and stored for subsequent use. The seeds were compared with museum specimens which had been collected in 1951 at the same location and which were the source of material used in other investigations (2, 3). The refrigerated and preserved fruits were sectioned with a sliding microtome in the usual manner and microscopical observations were recorded and/or photographed.

Leaves.—A branching shrub measuring up to four meters high, *Pyularia pubera* bears soft deciduous leaves arranged alternately with short petioles (rarely longer than 15 mm.). On the flowering branches the leaves are fewer in number and uniformly smaller than those on the nonflowering branches. The leaves are larger at the terminal end on both types of branches, but they gradually decrease in size, the smallest occurring at the axis of the branch with the main stem. The flowering branches usually bear from 7 to 10 leaves to a year's growth, the largest being about 12 cm. long and 5 cm. wide; the nonflowering branches may bear up to 15 leaves, the largest attaining a length of about 23 cm. and a width of 10 cm. The basal leaves may be as small as 9 mm. in length and 6 mm. in width. In contrast to the terminal leaves which are obovate-oblong and acute at both ends, the basal leaves are frequently elliptical and are rounded at both ends. Both types of leaves exhibit a marked veiny appearance and are somewhat pellucid-punctate. When young both stems and leaves are minutely downy, hence the specific name *pubera* which refers to the pubescence or hairy appearance. As the stems and leaves approach maturity some of the pubescence disappears but many soft hairs remain on the stems and on the veins and margins of the leaves.

Flowers.—The flowers are small and greenish, occurring in short terminal racemes (Fig. 1). They are separate-sexed, the staminate bearing a 4-, 5-, or 6-cleft calyx with recurved lobes and four, five, or six stamens on short filaments. The pistillate flowers are characterized by a pyriform (pear-shaped) inferior ovary surrounded or covered by the adherent calyx tube which is normally 5-parted. The author has observed a considerable number of flowers and fruits which exhibit a 4-, 5-, 6-, and even 7-parted calyx. Lawrence (12) stated that, in the *Santalaceae*, instead of a calyx tube the pistil is covered by a hypanthium-like cup, the perianth segments and stamens originating from or near its rim. This cup or disk enlarges with the maturation of the fruit and may be considered an identifying characteristic. Rarely more than five or six fruits are developed on one stem, the average number

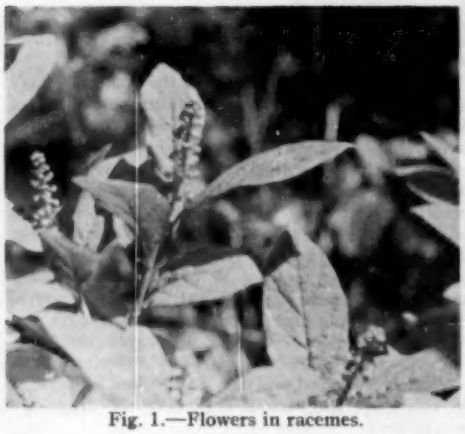


Fig. 1.—Flowers in racemes.

being two to four. The flowers appear in May, the young fruits are apparent in early June, and they are well formed by early July.

Fruits.—As the fruits approach maturity in early August they are fleshy and pyriform (Fig. 2) usually measuring 2.0 cm. in thickness and from 2.0 to 2.5 cm. in length, although occasionally the basal end of the fruit elongates into a stalk-like portion causing an over-all length of 3.0 cm. The surface is smooth and almost glabrous; the color is light green with small white raised areas near the basal end. At the apex of the mature fruit the hypanthium-like cup is very pronounced (Fig. 3), and the remains of the style, filaments of the aborted stamens, and the sepals or calyx lobes are all easily discernible. The recurved sepals are particularly characteristic and, as stated earlier, vary in number from 4 to 7. The apical end of the fruit is swollen around the sepals modifying the otherwise round disk. The fruits ripen in late August, gradually changing from green to yellow.

The pericarp is composed of a thin epicarp and a rather fleshy mesocarp that becomes compressed as the seed increases in size. In the immature fruit

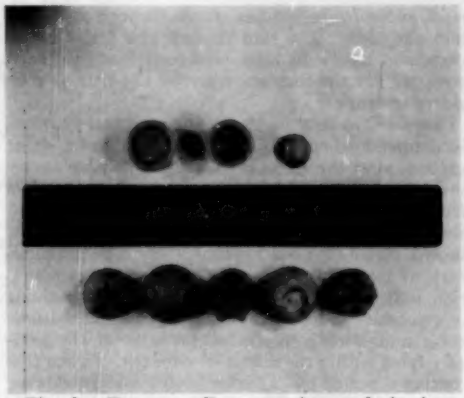


Fig. 3.—Top row: Transversely cut fruit showing from left to right—seed with endocarp intact, interior of apical end of fruit, and apical end of seed. Bottom row: Apical ends of five fruits showing 4-, 5-, and 6-lobed disks.

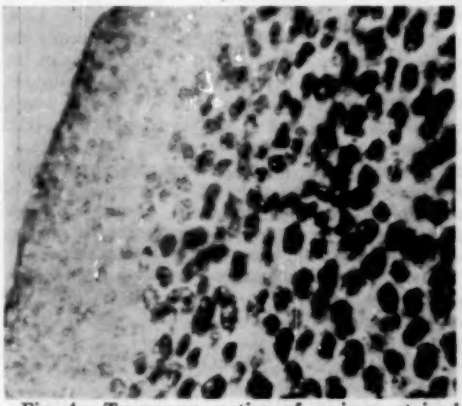


Fig. 4.—Transverse section of pericarp stained with iodine test solution showing the predominance of starch in parenchyma tissue of the mesocarp. ×440.

Fig. 2.—Pyriform-shaped fruits showing the 5-lobed hypanthium-like cup.

the endocarp is at first undifferentiated from the mesocarp except for a thin layer of brown-walled cells that are hardly noticeable. Within the space of three weeks this layer quickly develops into a well-defined, hard region that separates from the mesocarp and, thus, represents the endocarp (Fig. 3). After the fruit is shed, the pericarp (epicarp and mesocarp) splits lengthwise between the swollen calyx lobes to expose a single large seed with a smooth, hard covering which is the endocarp just described. This covering is light tan in color and can withstand considerable shock before it cracks. Subsequent microscopic examination shows it to be composed of lignified sclerotic tissue.

The immature fruits and seeds possess a slight, somewhat characteristic, unpleasant odor which becomes more pronounced at maturity. A bland oily taste also becomes apparent as ripening begins. Young fruits contain considerable starch in the mesocarp region (Fig. 4) but not in the developing

seed; older fruits contain starch in both mesocarp and the seed. The thin epicarp, the fleshy mesocarp, and the single large seed contained in a hard covering are the features whereby the fruit is considered a drupe.

Seed.—The seed measures approximately 1.5 cm. in diameter when fully developed. It has a slight projection at the apex (Fig. 3) and a definite cavity instead of a hilum at its base where it separates from the funiculus. Following its removal from the endocarp, the seed is further protected by a light brown, tissue-thin coating that adheres to the endocarp as well as to the seed. Microscopic examination indicates that this is not epidermal tissue, but is composed of parenchyma and vascular tissues; these undoubtedly represent the part of the pericarp lying within the sclerotic endocarp. When this coating is peeled off, a darker brown epidermal layer is seen beneath which is the endosperm tissue. Neither seed coats, cotyledons, nor embryo could be located. Although this lack of specialized organs may appear unusual, it can readily be explained. Goebel (13) indicated that, in the *Santalaceae*, the envelope of the rudimentary ovule is supplied by the ovarian wall and no seed coat is present. Lawrence (12) has also observed that in this family the ovule integuments are reduced to one or absent, the seed having no seed coat. Furthermore, in discussing seeds protected by the "stone" of the drupe, Strasburger, *et al.* (14), have stated that no special development of the seed coat occurs when its function is undertaken by the sclerotic endocarp; hence, it tends to become reduced. Regarding the absence of embryo and cotyledons, in some parasitic species and in most orchids, the embryo becomes apparent only after the seed begins to germinate. Therefore, further studies on the seed of *Pyrularia pubera* are necessary to understand its complete development.

HISTOLOGY

Fruit.—The pericarp is divided into the customary three layers of which the epicarp consists of one row of cutinized epidermal cells. In the immature fruits studied these average 22 μ in diameter. Beneath the epicarp are many rows of parenchyma containing fixed oil globules and storage contents. Small starch grains measuring 9 μ or less in diameter are compactly arranged in these cells, the grains being simple, rounded, and bearing a centric hilum with slightly discernible concentric lamellae.

Three interrupted rows of vascular traces and/or bundles occur throughout the pericarp of immature fruits. Two of these lie between the epicarp and the layer of brown-walled cells that later become the sclerotic region, the third row being interior to this region. The outermost row consists of spiral, scalariform, and occasionally pitted tracheae. These follow no definite directional pattern. The next row shows, in addition to vascular traces, occasional collateral bundles in which very small phloem cells lie exterior to the spiral and scalariform tracheae. The parenchyma cells of the mesocarp increase in size from the epicarp inward, the largest being in the area of the vascular bundles. The third row of vascular elements is located beneath the brown-walled cells and consists of spiral and scalariform tracheae interspersed among large, thin-walled parenchyma.

In the mature fruit the cells of the epidermis have increased in size, sometimes attaining a length of 50 μ and a width of 30 μ. Two rows of thick-walled hypodermal cells can be distinguished. The parenchyma of the mesocarp have become larger and are filled with starch grains (Fig. 4), oil globules, and protein matter. The sclerotic cells of the endocarp have developed from the layer of brown-walled cells and have become heavily lignified. These cells form a compact layer of from 10 to 12 rows and they sometimes measure 70 μ in length and 35 μ in width. The parenchyma and vascular tissues beneath the sclerotic tissue have become desiccated and form the thin cover of the seed partially adhering to the sclerotic region and partially to the seed epidermis. At this stage the various regions of the drupe are well defined.

Seed.—The epidermis of the mature seed consists of a single row of epidermal cells filled with a yellow content. Beneath the epidermis are large, rectangular, thin-walled cells presumably of the endosperm. They range from 50 to 70 μ long and from 35 to 50 μ wide, containing oil globules, starch, and protein matter. A few spiral and scalariform tracheae occur sporadically. Cotyledon and embryo tissues were not observed. The seed in this stage represents an almost undifferentiated mass of parenchyma tissue.

SUMMARY

1. The taxonomy, distribution, and habitat of *Pyrularia pubera* Michx. are described.

2. The morphology of the leaves and flowers is presented.

3. Structural changes are compared in the immature and mature fruits.

4. Morphological and histological features of the fruit and seed are recorded.

REFERENCES

(1) Wieczorkowski, J., and Darbaker, L. K., THIS JOURNAL, **22**, 45(1933).
(2) Danti, A., Sager, R. W., and Bianculli, J. A., *Am. J Pharm.*, **126**, 8(1954).
(3) Goldfield, R., Bianculli, J. A., and Sager, R. W., *ibid.*, **126**, 62(1954).
(4) Buckley, J. P., Coker, S. T., and Goldfield, R. (to be published).
(5) "Index Kewensis," Vol. 2, edited by J. D. Hooker and B. D. Jackson, Clarendon Press, Oxford, 1895, p. 668.
(6) "International Rules of Botanical Nomenclature," Science Press, Lancaster, Pa., 1935.
(7) Fernald, M. L., "Gray's Manual of Botany," 8th ed., American Book Co., New York, 1950, p. 561.
(8) Gleason, H. A., "The New Britton and Brown Illustrated Flora of the Northeastern United States and Adjacent Canada," Vol. 2, Lancaster Press, Lancaster, Pa., 1952, p. 59.
(9) Lyons, A. B., "Plant Names, Scientific and Popular," 2nd ed., Nelson, Baker and Co., Detroit, 1907, p. 386.
(10) Clute, W. N., "A Dictionary of American Plant Names," Willard N. Clute and Co., Joliet, Ill., 1923, p. 136.
(11) Demoise, C. W., *Pyrularia*, **1**, 25(1951).
(12) Lawrence, G. H. M., "Taxonomy of Vascular Plants," Macmillan Co., New York, 1951, p. 470.
(13) Goebel, K., "Organography of Plants," Pt. 2, Clarendon Press, Oxford, 1905, p. 619.
(14) Strasburger, E., *et al.*, "A Textbook of Botany," Macmillan and Co., London, 1912, p. 515.

A Study of Thermoelectric Methods for Determining Isotonicity*

By FRANK M. GOYAN and DONALD RECK†

It is proposed that the isotonicity of a given aqueous solution shall be expressed as the weight per volume percentage of a sodium chloride solution found by a study of suitable colligative properties to be isotonic with it. Methods developed during the last decade for the electronic amplification and recording of thermal electromotive force values in the microvolt range suggest the advisability of making increased use of thermocouples and thermopiles in the study of colligative properties. It is the purpose of this paper to discuss the application of thermoelectric methods with emphasis on various modifications of the Hill-Baldes techniques for determining isotonicity by detecting and measuring slight differences in temperature arising from differences in vapor pressure of thermally isolated samples exposed in constant humidity chambers. Several modifications of this technique are described, and the factors involved in the design of instruments for conveniently measuring isotonicity at any selected temperature are discussed.

SEVERAL years ago one of the authors became interested in the possibility of developing an instrument capable of indicating directly the osmotic or colligative properties of solutions. Thermoelectric methods (1) were selected for study in this connection because very small samples are required and because they are free from the temperature restrictions imposed by the freezing-point and boiling-point methods. The selection of these methods was also influenced by the development of d.-c. amplifiers sensitive in the microvolt range (2). This contribution of modern instrumentation is significant because thermoelectric methods are based upon the measurements of thermal electromotive force values which are proportional to small differences in the partial pressure of solvent over samples of solutions in thermal contact with thermocouple junctions. Recent work (3, 4) has indicated that thermistors may be substituted for thermocouple junctions in measuring the small differences in temperature developed by the condensation or evaporation of solvent from the otherwise thermally insulated samples.

Regardless of the measuring instruments selected, it would appear that the problem of designing a direct-reading "isotonicity meter" may be solved by drawing a suitable scale on a meter connected to the output terminals of a sensitive amplifier. Such a scale is important, regardless of the practicability of the thermoelectric method. This scale of values indicating osmotic concentration would serve to compare osmotic properties calculated from measurements of freezing points, boiling points, and osmotic pressures, and may be expected to clarify the concept of isotonicity for teaching purposes.

TERMINOLOGY AND VALUES

The Sodium Chloride Isotonicity Scale.—There are many logical ways of reporting the colligative properties of solutions for pharmaceutical purposes (5, 6). In 1945 Krogh, Lund, and Pedersen-Bjergaard (7) reported the osmotic concentration of human serum and lacrimal fluid in terms of NaCl solution by giving the value of 0.86% for serum and 0.90% for lacrimal fluid. These values seem much more meaningful than the corresponding freezing-point or osmotic-pressure values because they provide a basis for immediate direct comparison with physiological saline solution. Because of this fact, and because of the fact that solutions of NaCl constitute one of the established reference standards for the isopiestic method for determining activity coefficients (8, 9), it is proposed that the isotonicity scale shall be defined in terms of concentration of NaCl. This implies that an isotonicity value reported for an aqueous solution shall be the concentration of the pure aqueous solution of NaCl which can be shown to have identical colligative properties. The concentration of NaCl when used in this manner should be abbreviated in the customary way to distinguish between molar and per cent concentration. Thus the isotonicity of lacrimal fluid may be given as 0.90% NaCl or 0.154 *M* NaCl, whichever is more convenient. It may be assumed, unless otherwise stated, that both per cent (weight/volume as usually defined) and molar concentrations are based upon the final volume of solution at room temperatures near 25°.

Selection of Terminology.—The term *isotonic* has been used in pharmaceutical literature for many years to describe solutions which can be shown by measurement or by approximate methods of calculations (10) to have the same freezing-point depression, or other colligative property, as some standard solution selected for reference. Davson and Danielli (11) discuss the delayed hemolysis of erythrocytes caused by the slow penetration of the membrane by

* Received September 29, 1953, from the College of Pharmacy, University of California Medical Center, San Francisco.
Presented to the Scientific Section, A. PH. A., Salt Lake City meeting, August, 1953.
† Present address: 1528 W. Amerge, Fullerton, Calif.

glycerol from an isotonic solution of this substance. This usage is in agreement with the common usage discussed above. Husa and Rossi (12) state that the term isotonic is commonly considered to be synonymous with the term *isosmotic*. However, these workers call attention to the fact that the former term is ambiguous because it may or may not carry the additional implication of osmotic equilibrium across some specific animal membrane.

At first glance, it would seem desirable to use the less ambiguous synonym. This decision relegates the term isotonic to those special cases involving specific animal membranes. However, it is unlikely that advocates of this usage have weighed all of its implications, especially the inescapable conclusion that solutions which are isotonic with some accepted standard solution for one animal membrane are not necessarily isotonic for a different membrane. Another disadvantage of relegating the term isotonic to highly specialized biological systems relates to the fact that a transition from one usage to another causes great confusion. Unfortunately, this transition seems to be under way in many circles.

The proposed term *isotonicity value* may eliminate the growing confusion concerning the exact meaning of many of the calculations commonly used by pharmacists in adjusting solutions. It must be clearly understood that the proposed term excludes from consideration the properties of animal membranes. Fortunately, there is sufficient difference between the proposed terminology and the term isotonic that there need be no confusion resulting from a statement that all solutions having an isotonicity value of 0.90% NaCl are not necessarily isotonic solutions in a biological sense. However, the number of substances which approach this correlation is sufficiently large that it seems far easier to remember the solutes which are known to permeate or modify specific animal membranes than to attempt to include these exceptions in the terminology itself, especially in view of the fact that the rate of penetration of each different substance through each different membrane must also be considered.

Another good reason for adopting the term isotonicity is that the term isotonic is occasionally used as a synonym for the term *isopiestic* in the literature of physical chemistry (13). Experimentally determined isotonicity values may be used to calculate activity coefficients and other properties of solutions which could be determined by isopiestic methods. It is hoped that the term isotonicity will become as useful as the term *p*H in describing solutions. Like the latter term, it has the advantage that it may be used as a simple scale value which approaches linearity over short ranges and can serve as the basis for the design of direct-reading instruments. It permits the euphonious description of a solution as having, for example, a *p*H of 7.0 and an isotonicity of 0.90% NaCl.

Availability of Tabulated Isotonicity Values in Any System.—Fortunately, commonly available values for the sodium chloride equivalent of various solutes (10) are each numerically equal to the isotonicity value of the corresponding aqueous solution at unit concentration. A sodium chloride equivalent of 0.5, for example, implies that one weight unit of the substance under consideration is the osmotic equivalent of 0.5 of the same weight unit of NaCl; hence, a 1% solution of this substance may be assigned an isotonicity value of 0.5% NaCl. If it is clearly understood that the isotonicity value of a solution is defined as the concentration of a solution of pure NaCl which is isotonic with it, no confusion should result from the practice of assigning isotonicity values in grains per fluidounce, moles per liter, or other convenient concentration units. Although the same limitations and approximations are encountered with any system of values, there is considerable advantage in dealing with the osmotic properties of solutions in the system of units employed in expressing the results of calculations.

The approximate isotonicity calculations of practical pharmacy are easily visualized by the use of isotonicity values assigned on the basis of sodium chloride equivalents. The pharmacist must remember that an isotonicity value of 0.9 Gm. NaCl per 100 ml. or 4.1 grains NaCl per fluidounce is sought. He establishes any convenient volume unit, multiplies the weight of each solute dissolved in it by its sodium chloride equivalent to determine the isotonicity contribution of each, adds the resulting products, and compares the sum with the isotonicity value sought. If he is dealing with 4 fluidounces, for example, he may find it convenient to use this volume as his basic unit and to compare his sum with 16.4 grains NaCl per 4 fluidounces.

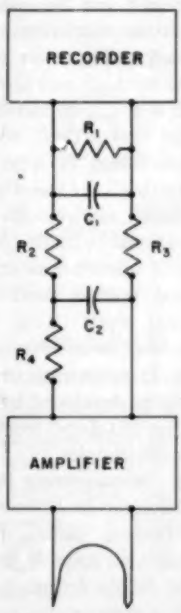


Fig. 1.—Electrical instrumentation: A Leeds and Northrup speedomax recorder (0–10 mv. scale) is coupled to the output of a d.-c. breaker amplifier (Model 70 by Liston-Folb, Stamford, Conn.) through the circuit shown in detail (R_1, 20; R_2, R_3, and R_4, 1000 ohms; C_1, 12; C_2, 240 m. f. d.). This circuit is not ideal, but gave satisfactory results. The copper-constantan thermocouple is described in the text. Input impedance characteristics make independent temperature calibration necessary.

EXPERIMENTAL

Instrumentation.—The electrical measuring instruments used in all but the exploratory phase of this work consisted of a Liston Folb D.-C. Breaker Amplifier coupled to a Leeds and Northrup Speedomax Recorder through an attenuator and filter circuit shown in Fig. 1. All of the thermocouples used were made from approximately No. 36 bare constantan and enameled copper wire. The junctions were made by twisting the two wires together and dipping the twisted ends into molten silver solder covered with a borax-type flux in an iron deflagrating spoon. After dipping into the solder, the wires were untwisted to the soldered joint and excess wire and solder cut away. Thermocouples used with the brass cylinders described below were painted with a glyptal type varnish. Those used with the glass tube assembly were slipped into fine glass capillary tubing just large enough to receive the wires and a fine capillary insulator. The outer capillary case was made as thin walled as possible and was sealed at the end where the junction was held in thermal contact with solidified naphthalene. The two capillaries were connected with a fine plastic tubing to protect the leads. The completed thermocouple had a resistance of about 20 ohms.

In the first series of experiments, brass cylinders about 20 mm. in diameter and 85 mm. tall were maintained in a constant temperature air bath. These cylinders had a wall thickness of about 3 mm. and were equipped with a flanged, two-hole bakelite cap machined so as to fit closely. The glass portion of a standard medicine dropper was slipped through each of the two holes in the caps. The tips of the droppers, thus suspended in the lower portion of the cylinders, served to hold samples and reference solutions. In all experiments the inside surfaces of the brass cylinders were covered with wet filter paper. This assembly was used by suspending loops of filter paper below each tip, moistening one loop with a reference solution and the other with a sample, and passing tiny thermocouple junctions through the medicine droppers so as to make contact with the moistened loops of paper. The spring suspension of the medicine droppers shown in Fig. 2 was necessary in order to maintain firm contact between the junctions and the filter paper.

In a few experiments, the filter paper was eliminated and the samples were held by capillary action in the tips. Each junction was shaped so that in passing through the sample it collected a tiny drop of liquid which remained suspended on the junction inside the brass cylinder.

A second series of experiments was set up to test the hypothesis that it is unnecessary to maintain the two junctions in the same vessel. The apparatus consists of three or more glass tube assemblies shown in Fig. 3. These tubes are suspended in water contained in a large covered beaker. The water is rapidly stirred but not supplied with regulated temperature control except through the air of the constant-temperature air bath. The temperature of the air is maintained by means of a fan and electrical heaters. Current through the heaters is controlled by a thyratron circuit (14) which responds to contacts made by a platinum wire soldered to the end of a flat spiral of bimetallic strip held so as to receive vibrations from the fan. This vibrating contact produces rapid surges of current through the heater, the total amount of heat being dependent upon the frequency and duration of the surges. This method of regulation was found necessary in order to avoid sudden changes in temperature caused by the conventional regulating systems. Constant refrigeration or constant additional heating is supplied, depending upon the temperature selected.

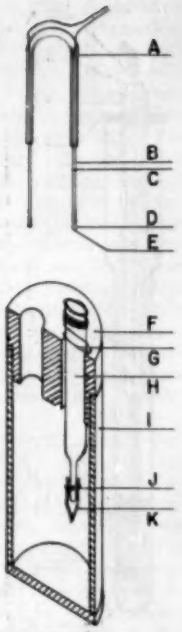


Fig. 2.—Brass cylinder assembly: A, glass tubing; *B*, copper wire; *C*, constantan wire; *D*, loop for holding sample or making contact with filter paper; *E*, junction; *F*, bakelite cap; *G*, spring; *H*, medicine dropper; *I*, Lacquered brass cylinder; *J*, rubber band; *K*, filter paper.

Results with the Brass Cylinder.—Results obtained with the brass cylinder as a constant humidity chamber at 30° are shown in Fig. 4. After several experiments it was found that the distance of the filter paper from the tip of the dropper was very important. If it was too close, the sample was not well isolated from the dropper and the difference between two different samples was neutralized to a great extent. A minimum of 4 mm. was found to be necessary. So a standard of 5 mm. was set and used. The width of the filter paper was set at 3 mm. Differences in final equilibrium were found between different cells using the same thermocouple unit. These differences were of the order of 0.1 microvolt. Using the same cell it is possible to obtain results differing by less than 0.02 microvolt. The degree of wetness of the filter paper loop and the time of exposure of the wet paper to the air of the thermostat before being placed in the cylinder were also found to be critical. However, it was possible to standardize the techniques without sacrificing the essential advantage of this method, namely, that samples may be collected by

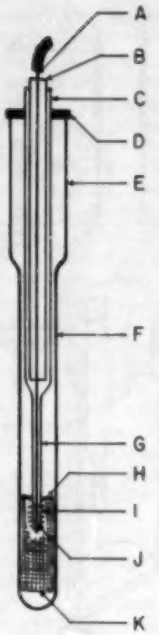


Fig. 3.—Glass tube assembly: *A*, removable junction incased in capillary (1 mm. x 15 cm.); *B*, removable junction shield; *C*, removable thermocouple well (8 mm. o. d.); *D*, rubber; *E*, transfer chamber (13 mm. o.d., 4 cm. long); *F*, part of outer tube in water bath (11 mm. o.d., 10 cm. long); *G*, capillary portion of thermocouple well (3 mm. o. d.); *H*, platinum foil disc; *I*, spiral of #36 platinum wire for holding 0.03–0.05 ml. of liquid; *J*, mercury; *K*, platinum gauze (2 wires per mm.) for holding reference liquid (cutaway drawing).

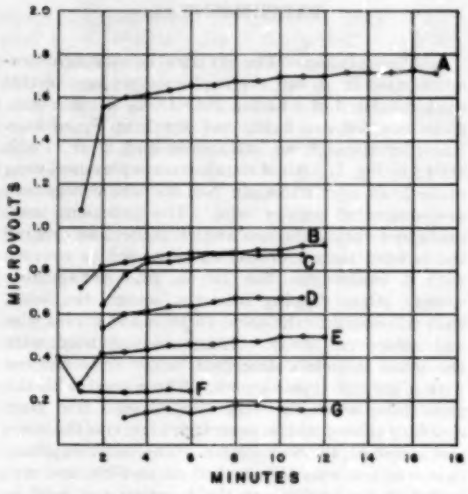


Fig. 4.—Summary of measurements at 30° on NaCl solutions with the brass cylinder and filter paper loop. Curve *A* represents 0.9% NaCl, curves *B–G* were obtained on essentially unknown solutions of NaCl which were later found to have the following concentrations by conductivity measurements: *B*, 0.514%; *C*, 0.504%; *D*, 0.406%; *E*, 0.306%; *F*, 0.126%; *G*, 0.109%. All measurements were made against water as the reference liquid.

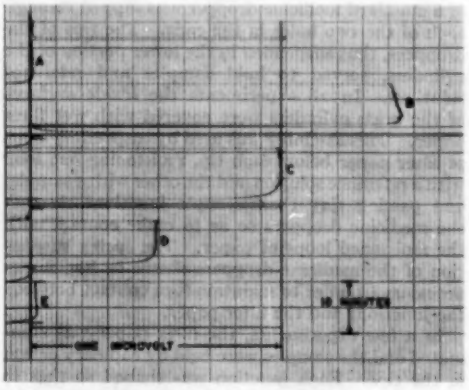


Fig. 5.—Photograph of a recording of measurements on NaCl solutions with the glass tube assembly at 25°. Curve *A* was obtained by transferring the thermocouple junction to a tube containing 0.9% NaCl on the spiral and also on the platinum gauze. This tube was placed in the bath about 10 minutes before zero time which corresponds to the bottom of the chart. Curves *B–E* represent successive samples placed on the spiral of a tube containing water on the gauze. The samples were: *B*, 0.9%; *C*, 0.6%; *D* 0.3% NaCl; and *E*, water.

simply dipping the dry paper loop in a liquid and quickly transferring it to the constant humidity chamber. It was found necessary to establish approximate limits for the tension of the spring holding the medicine dropper.

The results given in Fig. 4 show that it is reasonable to expect to design a thermoelectric instrument for determining isotonicity values without following the established techniques. Each of the curves of Fig. 4 has been reduced to a single value by estimating visually the microvolt reading of the best horizontal line through the last two or three points of each curve. When these values are plotted against concentration, the individual points deviate from linearity by less than 0.02% NaCl with two exceptions. The point derived from curve *D* is high by 0.02% NaCl, and the one from curve *F* deviates in the opposite direction by 0.04% NaCl. It is interesting to note that a satisfactory approximation of linearity is established within five minutes.

Results with the Glass Tubes.—Four tubes of the type shown in Fig. 3 were constructed. Two of these tubes, which were intended to be used only with water in order to establish a reference temperature, were made without the enlarged extension above the water bath. This extension serves merely to minimize the time of exposure of samples to the air of the thermostat and as a convenience when placing samples under the platinum spiral with a micropipet.

In all of the experiments with the glass tube assembly, the reference junction of the thermocouple

remained in the thermocouple well of the same tube. The platinum spiral of this tube held pure water and was surrounded by a platinum gauze also wet with water. The junction used for all of the samples was transferred from a duplicate temperature reference tube where it was placed to establish zero without the need for the carefully constructed electrical switches required for this type of work. The samples used in obtaining the recording shown in Fig. 5 were all introduced by drying the spiral with filter paper and dipping it into a bottle of the solution to be studied. The platinum foil shield serves to protect the sample from accidentally touching the inner surface of the tube as it is quickly lowered into position. The platinum gauze has a great advantage over filter paper in that it can be washed in cleaning solution, rinsed with distilled water and with any reference liquid required without removing it from the tube. Experiments with colored solutions show that this technique is adequate. Any tube may be lifted from the water bath without disturbing the others.

This apparatus was designed to prove that an exchange of vapor between a reference drop and a sample drop contained in the same chamber is not an essential feature of the thermoelectric method. The results displayed in Fig. 5 indicate that satisfactory measurements may be obtained when a single reference tube is reserved for the reference junction. Of course, this is achieved by good temperature control.

DISCUSSION

The recording reproduced as Fig. 5 shows that equilibrium is established quickly, and that microvolt readings may be regarded as a linear function of concentration. However, a slope in the direction of lower concentrations is observed with the more concentrated solution (curve *B* of Fig. 5). This phenomenon has been observed many times and is believed to be due to the dilution of the sample by condensing vapor. This explanation is supported by the fact that geometrical features of the tubes, the size of sample, etc., influence results to a greater extent as the difference in concentration between sample and reference liquid increases. However, concentration is not a serious limitation because of the facility provided for changing to more concentrated reference liquids, and the stability of the more concentrated samples under these conditions, as shown by curve *A* of Fig. 5.

These measurements may be thought of as determining the temperature of microsamples in approximate vapor-pressure equilibrium with a selected reference liquid maintained at a constant temperature. In this respect they differ from determinations of boiling-point elevations only by virtue of the fact that the partial pressure of air in the vapor phase is much higher.

The boiling-point constant for water at 25° may be estimated to be 0.33 (less than 0.51 in the ratio of the squares of the absolute temperatures). A 0.6% solution of NaCl is 0.1 *M* and approximately isotonic with an 0.18 *M* solution of an unionized solute. On this basis, a theoretical boiling-point elevation of 0.06° would be predicted for a 0.6% solution of NaCl at 25°. Direct calibration of the total circuit shown in Fig. 1 shows that a value of 1 microvolt as read from the test signal built into the amplifier is equivalent to 0.034°. Thus it appears from Fig. 5 that even with the thermocouple wells and rather crude adjustments employed it is possible to attain better than 50% of the maximum signal attainable with the most painstaking work in vacuum. This calculation indicates that future design should not sacrifice convenience of operation in order to attain the highly desirable objectives of minimizing heat conduction through thermocouple leads and shields, and increasing the rate of exchange of vapor between sample and reference liquid. However, it is important that the sample shall be introduced at a temperature as near that of the water bath as possible.

A comparison of curves *A* and *B* of Fig. 5 indicates that highest ultimate precision may be attained by developing methods based upon selecting, on the basis of preliminary approximations, reference solutions having isotonicity values close to the sample under investigation. This procedure would be expected to minimize errors caused by factors which alter surface conditions of the sample as well as those caused by variations in the size and position of the sample with respect to the reference solution.

One of the inherent limitations of any "isotonicity meter" based on the thermoelectric principle is that it will give anomalous results with solutions of volatile solutes. However, any thermocouple and amplifier suitable for any thermoelectric method can be used to obtain isotonicity values by the freezing-point method whenever the need arises.

REFERENCES

(1) Weissberger, A., "Physical Methods of Organic Chemistry," Vol. I, Part I, 2nd ed., Interscience Publishers, Inc. New York, 1949, p. 546.
(2) Liston, M. D., Quinn, C. E., Sargeant, W. E., and Scott, G. G., *Rev. Sci. Instr.*, **17**, 194(1946).
(3) Müller, R. H., and Stolten, H. J., *Anal. Chem.*, **25**, 1103(1953).
(4) Huff, H., McBain, J. W., and Brady, A. P., *J. Phys. & Colloid. Chem.*, **55**, 311(1951).
(5) Szekely, I. J., and Goyan, F. M., THIS JOURNAL, **41**, 30(1952).
(6) Martin, A. N., and Sprowls, J. B., *Am. Profess. Pharmacist*, **17**, 540(1951).
(7) Krogh, A., Lund, C. G., and Pedersen-Bjergaard, K. *Acta Physiol. Scand.*, **10**, 88(1945).
(8) Robinson, R. A., and Harned, H. S., *Chem. Revs.*, **28**, 419(1941).
(9) Harned, H. S., and Owen, B. B., "The Physical Chemistry of Electrolytic Solutions," Reinhold Publishing Corp., New York, 1943, p. 289.
(10) Szekely, I. J., Goyan, F. M., and Hind, H. W., THIS JOURNAL, **41**, 32(1952).
(11) Davson, H., and Danielli, J. F., "The Permeability of Natural Membranes," Macmillan Co., New York, 1943 p. 24.
(12) Husa, W. J., and Rossi, O. A., THIS JOURNAL, **31**, 270(1942).
(13) Glasstone, S., "Thermodynamics for Chemists," D. Van Nostrand Co., Inc., New York, 1947, p. 387.
(14) Swinehart, D. F., *Anal. Chem.*, **21**, 1577(1949).

Derivatives of Alginic Acid*

By J. E. WINTTER,† W. M. LAUTER,‡ and P. A. FOOTE§

Alginic acid and mononitrated alginic acid were reacted with *n*-amyl-iodide, β-diethylaminoethylchloride, *n*-butylchlorosulfite, and *n*-amylchlorosulfite, but failed to produce significant amounts of esterification in each case. Mixed formyl, acetyl, propionyl, and butyryl esters of nitrated alginic acids were prepared by progressive replacement of the nitrate groups, employing formic acid or the respective acid anhydrides. The reaction of styrene oxide as esterification agent showed only an 8–12 per cent yield. The reaction between alginic or mononitrated alginic acid with diazoacetic ester produced various degrees of esterification. While the alginic acid gave only ester yields of 8 per cent, nitrated alginic acid, probably due to its better solubility properties, produced esterification on the order of 70 per cent. The instability of nitrated alginic acid and alginic acid when subjected to most esterification procedures suggests the diazoacetic ester method as especially well suited for the introduction of specific groups into nitrated alginic acid.

DERIVATIVES of alginic acid are increasing in commercial importance, largely because of their hydrophilic-colloidal properties. The use of alginic acid to control the actions of certain drugs through control of viscosity, thereby regulating absorption, assimilation, and physiological action of the respective drugs, has been suggested (1). Esterification of this long-chain anhydro-D-mannuronic acid presents difficulties, because of the instability of the chain when exposed to most standard esterification procedures. Lucas and Stewart (2) were able to introduce up to 1.4 methoxyl groups per mannuronic residue without degradation, by using diazomethane in ether. Dimethylsulfate was impractical because of degradation of the acid. Various acetates of alginic acid have been prepared (3, 4, 5, 6).

Attempts to obtain acetone-soluble derivatives by the action of benzoylchloride and phenacylbromide on sodium alginate were not successful (2). Steiner and McNeely prepared alkyleneglycol esters of alginic acid by treating it with alkylene oxides in the presence of water (7, 8, 9). The investigations of Schneider and Ziervogel (10) showed that the progressive replacement of nitrate groups in nitrated pectinic acids by means of formic acid and acetic anhydride, respectively, produced mixed formyl-nitrate and acetyl-nitrate esters of pectinic acids. In view of the structural relationship of those acids to alginic acid it was felt that this reaction would be of value in our studies.

The diazoacetic ester method of esterification appears not to have been investigated previously in the field of uronic acids, and hence offerred an interesting possibility.

The alginic acid used in this study was supplied by the Kelco Company of San Diego, California, under the designation Kelacid (S.S.-621-3). It was a light, white powder, sufficiently fine for 75 per cent of it to pass through a No. 100 sieve. In terms of viscosity, a 3 per cent solution in an excess of ammonium hydroxide had a medium viscosity (ca. 270 c. p. s. at 25°).

A sodium alginate (Kelcosol,® KL-94830) of high viscosity was also used in this investigation. The alginic acid had a moisture content by toluene distillation method of 12.1 per cent; total ash (dry basis) 2.45 per cent; neutralization equivalent 188.8 (av.), theoretical 176; *p*H 2.6 for a 1 per cent aqueous dispersion. Steiner (11) found the ash to consist mostly of carbonates of calcium and sodium as well as some chlorides.

EXPERIMENTAL

Action of Aliphatic and Substituted Aliphatic Alkyl Halides on Alginic Acid.—Alkylation of alginic acid by the action of alkyl halides has been largely confined to methylation. We applied this method to three other halides. Saponification tests were made in the usual manner on the solid material which remained after each of these reactions. A blank determination using sodium alginate was made in the same way. The general esterification method employed either sodium or diethylamine alginate which was refluxed with the alkyl halide, in a suitable organic solvent, for several nours with continuous stirring. The reaction product was then separated and its esterification percentage determined. Table I shows that no significant esterification could be obtained by this method.

Alkylchlorosulfites as Esterification Agents for Alginic Acid.—Since alkyl halides failed to produce significant esterification it was of interest to evaluate the method by Newman and Fones (12) developed in 1947. *n*-Butylchlorosulfite and *n*-amylchlorosulfite were used. The sodium alginate used was dried at 60° *in vacuo* over concentrated sulfuric acid. As a solvent, dry benzene was used.

Sodium alginate (6.6 Gm.) was suspended in 75

* Received August 14, 1954, from the College of Pharmacy, University of Florida, Gainesville.
Abstracted from a dissertation presented to the Graduate School of the University of Florida by John E. Wintter in partial fulfillment for the degree of Doctor of Philosophy.
† Fellow of the American Foundation for Pharmaceutical Education, now Associate Professor at Howard College, Birmingham, Ala.
‡ Professor of Pharmaceutical Chemistry, University of Florida.
§ Dean, College of Pharmacy, University of Florida.

TABLE I.—ESTERIFICATION OF SODIUM OR DIETHYLAMINO ALGINATES

Alkyl Halide, Gm.	Salts of Alginic Acid, Gm.	Yield, Gm.	Esterification, %	Solvent, cc.	Time of Refluxing, Hrs.
n-Amyliodide, 24	Sodium alginate, 24	24.4	2.04 2.00 2.17 Av. 2.09 (expressed as amyloxy-)	Benzene, 200	5
β-Diethylaminoethylchloride-hydrochloride, 8.6	Sodium alginate, 24	46.8	3.18 3.20 3.19 Av. 3.18 (expressed as β-diethylaminoethoxy-)	Toluene, 150	3
β-Diethylaminoethylchloride, 6.8	Sodium alginate, 4.9	4.5	4.86 4.75 4.79 Av. 4.80 (expressed as β-diethylaminoethoxy-)	Isopropanol, 300	5
β-Diethylaminoethylchloride-hydrochloride, 10.3	Diethylamino alginate (77% neutralized), 15.8	3.5	No esterification	Propyleneglycol,[a] 300	2 (at 120°)
Ethylene chlorhydrin, 64.4	Sodium alginate, 19.8	20.8	5.50 5.40 5.50 Av. 5.47 (expressed as HO—CH_2—CH_2—O—)	None	4

[a] Propyleneglycol is a solvent at 120° for both reactants. Degradation of alginic acid was observed.

cc. of dry benzene. Ten grams of *n*-butylchlorosulfite, dissolved in 15 cc. benzene, was added dropwise with continuous stirring. Contrary to the observations of Newman and Fones, no vigorous exothermic reaction occurred. The mixture was then refluxed for two hours. The benzene was then removed by distillation. Sulfur dioxide was evolved at 130°. After five hours at that temperature no further sulfur dioxide was formed. After extraction of the product with benzene, a residue of 5.9 Gm. was obtained. It was insoluble in the usual organic solvents, but was water soluble. Saponification tests using sodium alginate as a blank gave an esterification percentage of only 3.29% (expressed as butyloxy-). Repetition of this experiment, but employing *n*-amylchlorosulfite, failed to show any evidence of esterification.

Acylation of Nitrated Alginic Acid.—Since one may assume that the difficulties in esterification of alginic acid stem largely from its very low solubilities in most solvents, it was felt that the more soluble nitrated alginic acids might offer better possibilities.

Nitration of alginic acid was accomplished by two different procedures. The method by Speiser and Eddy (13) was found to be preferable to that of Lucas and Stewart (2). The former nitrated pectin at 0° with fuming nitric acid.

Anhydrous alginic acid was added, while stirring vigorously, to a cooled fuming nitric acid solution (d. 1.5) at 0°. A temperature from 0–5° was maintained for at least one hour under constant agitation. Unlike pectin, the alginic acid did not dissolve in the nitric acid during this time. The reaction mixture was poured into a large volume of water, and cooled to 10°. After stirring for a few minutes, a flocculent precipitate settled which was filtered and washed with water at 10°. The crude product was purified by dissolving it in acetone, centrifuging off all insoluble alginic acid, and reprecipitating the nitrated alginic acid with ether, chloroform, or toluene. The reaction products obtained by the various modifications employed by us and dried over P_2O_5 are shown in Table II.

TABLE II.—NITRATION OF ALGINIC ACID AT 0–5°

	Experiment 1	2	3	4	5
Alginic acid, Gm.	5.0	10.4	20.7	23.0	25.0
Nitric acid (d. 1.5), cc.	166	333	666	666	666
Nitration, hrs.	1.5	2.0	2.0	4.0	2.0
Yield, Gm.	4.4	11.6	25.2	28.5	31.8
Nitrogen, %	5.90	6.35	6.23	6.03	5.42
Neutralization equivalent	218	229	236	230	220

The data indicate that approximately one nitrate group has been added per mannuronic residue. This differs from the 1.6–1.8 nitrate groups per residue found by Speiser and Eddy (13) in the case of pectin. The yields are quite satisfactory.

Good solvents were: methanol, ethanol, acetone, tetrahydrofuran, 5% aqueous Na_2CO_3 solution. Fair solvents were: methylethyl ketone, dioxane, pyridine, ethylacetoacetate. Poor solvents were: ethyl acetate, amyl acetate, glacial acetic acid. The nitrated alginic acids were insoluble in water, ether, chloroform, benzene, ethylenedichloride.

Nitrated alginic acid, if left at room temperature, tends to decompose as evidenced by formation of some NO_2 fumes. It is stable, however, at 0–4°.

The type of nitrated alginic acid as described under Experiment 4 in Table II was used following the method by Schneider and Ziervogel (10) in the field of nitro-pectins. This method partially replaces nitrate groups and forms mixed esters of nitro-pectins.

The acylation of nitrated alginic acid was carried out with formic acid, acetic anhydride, propionic acid anhydride, and butyric acid anhydride, using the following general procedure.

The alginic acid derivative (6.03% nitrogen) was dissolved cold in an excess of the acylation reagent in the presence of a small quantity (1% w/v) of concentrated sulfuric acid. The mixture was stirred until all the nitrated alginic acid had disappeared. Ten cubic centimeter aliquots, except for propionic acid anhydride and butyric acid anhydride (Table IV), were removed every twenty-four hours, diluted with an equal volume of acetone, and extruded into a large volume of water with vigorous agitation. The reaction products were separated by centrifuging. The supernatant liquid was decanted and ether added until precipitation was complete. Both the methanol-insoluble and the methanol-soluble portions were collected, dried, and analyzed (Table III).

In the case of propionic acid anhydride and butyric acid anhydride, solution of the nitrated alginic acids took from nine to thirteen days.

The crude reaction product obtained by precipitation with water was isolated, dried, and weighed. The fraction of this crude product soluble in acetone was then determined after evaporation of the solvent. Only the acetone-soluble fraction was assayed.

Esterification of Alginic Acid with Styrene Oxide.—Steiner (7) found that water must be present in the action of alkylene oxides on alginic acid or partially neutralized alginic acid.

Since styrene oxide and its ring substitution derivatives offered interesting possibilities, and is a readily available commercial product, esterification was attempted by the Steiner method. Ten grams alginic acid, 1.2 Gm. water (carried by the alginic acid), 1.8 Gm. water (added), 20.0 Gm. acetone, and 25.2 Gm. styrene oxide were reacted by adding the styrene oxide dropwise. An exothermic reaction, sufficiently strong to cause refluxing, was noticed. After keeping the reaction mixture for nine hours at 50–55° while stirring continuously, an equal volume of acetone was added. The insoluble material was filtered off and washed with acetone until free from styrene oxide. The yield of dried white material was 11.7 Gm. It was insoluble in water or the common organic solvents. Neutralization and saponification data showed 91.4% free acid and 8.6% ester.

When a portion of this material was subjected to a second treatment with styrene oxide exactly as described above, no solubility change in the resulting material could be observed. The neutralization and saponification data showed only a small increase in ester percentage, *viz.*, from 8.6 to 12.1%.

It was hoped that better results might be obtained by employing partially neutralized alginic acid as suggested by Steiner (8). The greater solubility of a 25% Na_2CO_3 neutralized alginic acid, because of better water solubility, made this change in procedure logical.

From 10.3 Gm. alginic acid (neutralized with Na_2CO_3 to 25% of theoretical amount) a yield of 15.7 Gm. of a white substance was obtained, insoluble in the common organic solvents as well as water. Its assay showed that it contained 63 *M* % free acid, 12 *M* % ester, and 25 *M* % salt.

This method does not appear to be satisfactory for our purposes, and we investigated instead the possibilities of applying the diazoacetic ester procedure to alginic acid.

Esterification of Alginic and Nitrated Alginic Acid with Diazoacetic Ester.—Diazoacetic ester is known to react with organic acids to give esters. One-half gram alginic acid (anhydrous) and 2.0 Gm. diazoacetic ester were mixed for one hour at 55–60°.

TABLE III.—ACYLATION OF NITRATED ALGINIC ACID WITH FORMIC ACID AND ACETIC ANHYDRIDE

Acylation Reagent, cc.	Nitrated Alginic Acid, Gm.	Time of Reaction, Hr.	Methanol-Soluble Material			Methanol-Insoluble Material		
			Yield, Gm.	Nitrogen, %	Neutralization Equivalent	Yield, Gm.	Nitrogen, %	Neutralization Equivalent
Formic acid[a] (88%), 45	5	4	0.6	5.53	230	...	...	...
		48	0.55	4.80	234	...	...	...
		72	0 07	4.61	225	0.27	2.54	229
		96	...	...	...	0.40	2.01	235
Acetic anhydride,[b] 37	5	24	0.35	5.08	266	0.11		
		48	0.27	3.26	259	0.12		
		72	0.25	2.45	261	0.13		
		96	0.30	2.40	264	0.12	Not determined	Not determined
		120	0.28	2.20	270	0.13		
		144	0.31	1.99	268	0.10		

[a] Formylated nitrated alginic acids differed from original nitrated alginic acids by swelling in water, partially dissolving in aqueous acetone, and being insoluble in the organic solvents referred to above.

[b] The acetylated nitrated alginic acids swelled in water and showed less solubility in the above-mentioned organic solvents than the original nitrated alginic acid.

TABLE IV.—ACYLATION OF NITRATED ALGINIC ACID WITH PROPIONIC ACID AND BUTYRIC ACID ANHYDRIDES

Acylation Reagent, cc.	Nitrated Alginic Acid, Gm.	Aliquot of Total Volume Used	Yield of Crude Product, Gm.	Acetone-Soluble Material		Neutralization Equivalent
				Yield, Gm.	Nitrogen, %	
Propionic[a] acid anhydride, 35	5	50% after 9 days	2.96	1.56	2.46	275
		50% after 10 days	3.75	2.37	2.32	279
Butyric[b] acid anhydride, 35	5	10 cc. after 9 days		0.70	4.49	280
		10 cc. after 10 days		0.65	4.08	282
		10 cc. after 13 days		0.66	3.80	280

[a] The acetone-soluble material had only a slight tendency to peptize in water.
[b] Solubility of acetone-soluble material is similar to that of the original nitrated alginic acids in acetone, dioxane, ethyl acetate, and tetrahydrofuran.

Nitrogen bubbles formed in the suspension. After standing for fifteen hours at room temperature, ether was added, and the material filtered and washed with ether until free from yellow color. Yield: 0.49 Gm.; per cent esterification (expressed as $-O-CH_2-\overset{\overset{O}{\|}}{C}-O-C_2H_5$): 4.42 (av.); theoretical, 35.93%.

The reaction product was insoluble in water or common organic solvents, but dissolved readily in Na_2CO_3 solution.

It is again the difficulty of the insolubility of the alginic acid in nearly all solvents which not only in this case, but in most esterification methods, poses such obstacles, and which does not permit a positive evaluation of any particular method in the case of alginic acid. Hence, we felt obliged to employ again nitrated alginic acid for this reaction. No replacement of nitrate groups is involved in this procedure. One-half gram of nitrated alginic acid (5.42% nitrogen) was dissolved in 1.5 Gm. diazoacetic ester, giving a viscous solution. Evolution of nitrogen started at once. After one hour at 55–60°, followed by standing overnight, a mixture of equal volumes of ethyl acetate and ether was added until no further precipitation could be observed.

After filtering, washing several times with ether, and drying, a yield of 0.46 Gm. was obtained. This compound is less soluble in ethanol and acetone and more soluble in methylethyl ketone than the original nitrated alginic acid.

Assays.—Per cent nitrogen, found: 4.92 (average); % nitrogen, nitrated acid: 5.42, neutralization equivalent, 842; % free carboxyl, 5.42; % free carboxyl for theoretical alginic acid mononitrate, 20.36; mole % ester, 72; mole % free acid, 28. Since alginic acid itself is insoluble in an excess of diazoacetic ester, but the esterified product might be soluble in organic solvents, ethyl acetate was chosen as solvent in a repetition of this reaction. Nine-tenths gram anhydrous alginic acid, 1.1 Gm. diazoacetic acid ester, and 50 cc. ethyl acetate were heated to 60° for eight hours under constant agitation. The alginic acid did not dissolve, and the neutralization equivalent of the isolated residue was 264, while the theoretical value for nitrated alginic acid is 221 (mononitrate). Saponification tests indicated a low ester percentage of 12. This shows that a decided excess of diazoacetic ester is essential to obtain good esterification of nitrated alginic acid as shown above.

SUMMARY AND CONCLUSIONS

1. Esterification of alginic acid and mononitrated alginic acid failed to produce significant amounts of esters when the acids were reacted with *n*-amyliodide or β-diethylaminoethylchloride. Equally unsatisfactory was the esterification method using two alkylchlorosulfites (*n*-butyl and *n*-amylchlorosulfite).

2. Nitration of alginic acid used in these experiments resulted essentially in the mononitrate of alginic acid which proved to be in the form of the free acid and not as a lactone.

3. Styrene oxide produced only a small amount of esterification (8–12 per cent), although very definite signs of reaction were noticed.

4. Mononitrated alginic acid gave formyl, acetyl, propionyl, and butyryl esters of partially nitrated alginic acid by progressive replacement of the nitrate groups of the acid.

5. Diazoacetic ester reacted readily with mononitrated alginic acid producing esterification on the order of 70 per cent. Alginic acid itself, probably because of poor solubility characteristics, gave only a small amount of ester.

REFERENCES

(1) Editorial, *Bull. Am. Soc. Hosp. Pharm.*, **8**, 397(1951).
(2) Lucas, H. J., and Stewart, W. T., *J. Am. Chem. Soc.*, **62**, 1070(1940).
(3) Gomez, M. L., *Inst. espan. oceanog. Notas y resúmenes, Ser. II*, **74**, 98(1933); through *Chem. Abstr.*, **28**, 2701(1934).
(4) Barry, V. C., Dillon, T., and O'Muineachin, P., *Sci. Proc. Roy. Dublin Soc.*, **21**, 285(1936); through *Chem. Abstr.*, **30**, 4161(1936).
(5) Wassermann, A., *Nature*, **158**, 271(1946).
(6) Chamberlain, N. H., Cunningham, G. E., and Speakman, J. B., *ibid.*, **158**, 553(1946).
(7) Steiner, A. B., U. S. pat. 2,426,125, 1947.
(8) Steiner, A. B., and McNeely, W. H., U. S. pat. 2,494,912, 1950.
(9) Steiner, A. B., and McNeely, W. H., U. S. pat. 2,494,911, 1950.
(10) Schneider, G., and Ziervogel, *Ber.*, **69**, 2530(1936).
(11) Steiner, A. B. (Kelco Co., San Diego, Cal.), persona communication.
(12) Newman, M. S., and Fones, W. S., *J. Am. Chem. Soc.*, **69**, 1046(1947).
(13) Speiser, R., and Eddy, L. R., *ibid.*, **68**, 287(1946).

The Effect of Germination on the Alkaloid Content of Nigella damascena Seed*

By V. E. TYLER, JR.†

A sensitive fluorometric procedure for the quantitative determination of damascenine was developed. Application of the method to the viable seeds and to the germinated seedlings and seed coats of *Nigella damascena* revealed that the alkaloid is metabolically inert. It failed to disappear from the testas during germination and was not formed in the etiolated seedlings. Paper partition chromatography was utilized to verify the identity and homogeneity of damascenine.

DAMASCENINE, the alkaloid of the seed of *Nigella damascena* L. has been defined chemically as 3-methoxy-N-methylanthranilic acid methyl ester (1). It is of particular interest from the biosynthetic viewpoint because of its structural relationship with 3-hydroxyanthranilic acid, a compound of recognized importance in the intermediary metabolism of certain microorganisms (2). This experiment was carried out to determine if damascenine is capable of re-entering metabolism during germination of *N. damascena* seed, or if the methylation of the substituent groups had rendered the compound metabolically inert. The study appeared especially feasible since damascenine is the only alkaloid found in the seed (1).

EXPERIMENTAL

Isolation.—Damascenine was isolated from 800 Gm. of the coarsely powdered seed of *N. damascena* according to the method of Ewins (1). Redistillation of the product under reduced pressure resulted in a yield of 1.35 Gm. (0.17%). The identity of the alkaloid was verified by the preparation of two derivatives. Damascenine picrate, prepared as described by Ewins (1), had a micro m. p., K. = 160–161.5°. The chloroplatinate derivative prepared according to the method of Keller (3) had a micro m. p., K. = 188.5–189°.

Quantitative Determination.—Since damascenine exhibits an intense blue fluorescence when dissolved in organic solvents, it was decided to utilize fluorometric analysis as a means of quantitative determination. For this purpose a Fisher Nefluoro-Photometer equipped with a mercury light source, center filter #365 and right and left filters #430+, was employed. The fluorescent generator solution consisted of an alcoholic solution containing 50 μg. per ml. of anthranilic acid and fluorescence was adjusted to 100% for a standard alcoholic solution containing 6.25 μg. of damascenine per ml. A standard curve was prepared from the fluorescence of alcoholic solutions containing 5.0, 3.0, 1.56, and 0.78 μg. of damascenine per ml. (Fig. 1.)

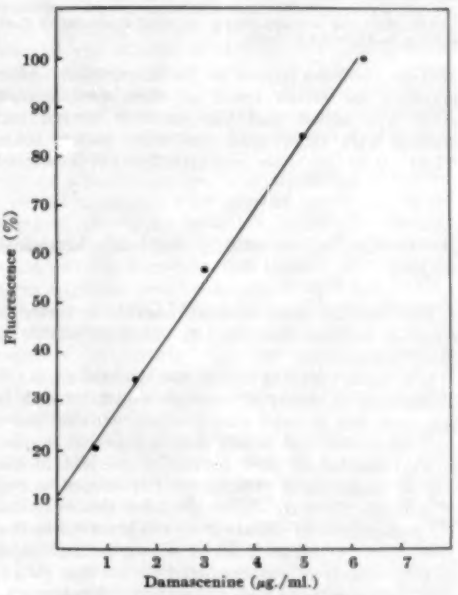


Fig. 1.—Damascenine standard curve

A procedure was then developed for the quantitative extraction of damascenine from plant material. The seeds or seedlings to be assayed were placed in a small porcelain mortar and triturated with a few drops of alcohol to a smooth pasty mass. Alcohol was added dropwise and trituration continued until the plant material was completely disintegrated. The extract, together with suspended particles, was transferred quantitatively by washing with alcohol to a small glass percolator (1 x 10 cm.), which contained a tightly packed pad of glass wool. Maceration was continued in the percolator for at least one hour and followed by slow percolation to the requisite volume (25 or 50 ml.). Aliquots of the solution were measured fluorometrically as previously described.

Since damascenine fluoresces only as the free base, the alcoholic extracts were checked routinely to insure against acidity. The *p*H of all extracts approached neutrality and the intensity of fluorescence was not increased by the addition of a drop of ammonium hydroxide. Alcoholic solutions of damascenine appeared to be quite stable and showed no quantitative change in fluorescence when measured repeatedly at twenty-four-hour intervals.

Eight groups of 10 seeds each were selected at random, weighed on an analytical balance, extracted,

* Received July 1, 1954 from the College of Pharmacy, University of Nebraska, Lincoln.
† Associate Professor of Pharmacognosy, University of Nebraska, College of Pharmacy.

TABLE I.—RESULTS OF ASSAYS FOR DAMASCENINE

Sample (10 units each)	No. of Samples	Sample Wt., mg., ± s	Fluorescence, %	Damascenine per ml., μg.	Dilution Factor	Damascenine in Sample, μg., ± s
Before Germination						
Whole seeds	8	28.4 ± 1.5	76.6	4.512	50	225.6 ± 12.0
Kernels	6	22.3 ± 1.2	11.7	0.108	25	2.7 ± 2.2
Seed coats		Calculated by difference (225.6 μg. − 2.7 μg.) =				222.9
After Germination						
Seed coats	8	7.7 ± 0.4	78.6	4.647	50	232.2 ± 9.9
Seedlings	8	21.3 ± 0.7	3.3	0	25	0

and assayed as described. The results are listed in Table I. The average content of damascenine corresponded to about 0.79% of the weight of the seeds, a figure somewhat higher than the 0.5–0.6% of damascenine hydrochloride isolated by Keller (4), but in satisfactory agreement when the differences in source of material and extraction procedures are considered.

The assay procedure yielded negative results for damascenine when applied to the seeds of *Nigella sativa* L.[1] This agreed with the results obtained by Keller (4), who was unable to detect damascenine in the seeds of this species.

Localization.—Microchemical tests have yielded conflicting results in regard to the site of alkaloid accumulation in *N. damascena* seed. Various investigators have reported the existence of the alkaloid in the seed coat, the outer layers of endosperm or the entire seed. Another has been unable to confirm the presence of alkaloid (5). A study was undertaken, therefore, to determine the quantitative distribution of damascenine in the seed before proceeding with germination studies.

A quantity of seed was soaked in water for several hours and the testas removed with tweezers. The process proved to be difficult because of the small size of the seeds. Although the testas were completely removed from the kernels, they could not be collected quantitatively. Therefore the kernels only were reserved for assay and the amount of alkaloid in the seed coats determined by difference.

The kernels were dried in a vacuum desiccator over calcium chloride for two weeks and allowed to equilibrate in air for an equal period of time. Subsequently they were divided at random into six groups of 10 each, weighed, extracted and assayed as described. The results listed in Table I indicate that approximately 1.2% of the total damascenine present in the seed occurs in the kernel. It thus appears that nearly all of the alkaloid occurs in the testa.

Germination.—A quantity of *N. damascena* seed was germinated in moist air according to the procedure described by Meyer and Anderson (6). The temperature during germination was regulated at 28–30° and the seeds were kept in the dark. Germination was allowed to continue until the seedlings approached maturity and the seed coats separated easily from the cotyledons. This time varied somewhat with individual seeds but averaged about ten days.

The etiolated seedlings and seed coats were collected at random in groups of 10 and dried in a vacuum desiccator over calcium chloride for a minimum period of forty-eight hours. Following this, the materials were equilibrated in air for an equal period of time, weighed, extracted, and assayed. The results are recorded in Table I.

Damascenine was not utilized during germination since fully as much alkaloid was recovered from the separated seed coats as was shown to exist in the viable seed. In fact, the average for eight determinations of groups of 10 each showed a slightly higher alkaloid content in the seed coats than in the original seed. To determine if this apparent increase was statistically significant, the t test was applied to the two means as described by Youden (7). The t value so obtained was 1.20 with 14 degrees of freedom which showed a probability level of approximately 0.25, indicating that the difference between the two means was not significant.

Damascenine neither increased nor decreased during germination but remained metabolically inert. No alkaloid was detected in the seedling.

Chromatographic Analysis.—Paper partition chromatography was utilized to verify the identity of the fluorescent material in the seeds before and after germination. Strips of Whatman No. 1 filter paper, 1 in. x 22.5 in. were dipped in 0.1 *M* phosphate buffer at *p*H 8, prepared from equal quantities of 0.1 *M* monosodium phosphate and 0.1 *M* disodium phosphate and adjusted to the indicated *p*H by addition of a saturated solution of sodium hydroxide. The strips were then allowed to air-dry. Ten microliter quantities of an alcoholic solution of damascenine containing 0.25 μg./μl. were spotted and the strips formed by the ascending technique. The wash liquid used consisted of the upper layer of a mixture of *n*-butanol and water (50-50). Time of formation approximated twenty-four hours. The alkaloid could be detected readily on the strip by examination under ultraviolet light.

Under these conditions damascenine showed an R_f value of 0.98 and was readily separated from closely related compounds such as anthranilic acid and 3-hydroxyanthranilic acid which exhibited R_f values of 0.63 and 0.70 respectively.

Concentrated alcoholic extracts of the viable seeds and of the seed coats separated after germination were chromatographed by this procedure. Only one fluorescent substance was located on the chromatograms. It possessed an R_f of 0.98, was inseparable from, and assumed to be damascenine.

DISCUSSION

The seeds of *N. damascena* exhibit unusual behavior during germination in comparison to other alkaloid-containing seeds belonging to the same

[1] Supplied through the courtesy of S. B. Penick and Co.

general class. According to James (8), those seeds which contain alkaloids in the peripheral tissues undergo a loss from those tissues during germination. Frequently, the alkaloid completely disappears from the seed, but detectable quantities reappear in the meristems of the seedling at a very early stage, in some instances when the radicle is only 2 mm. long. *N. damascena* seeds fail to conform to this pattern. Although the vast majority of damascenine is contained in the testa, the quantity fails to diminish on germination; moreover, detectable quantities of alkaloid are not formed in the mature etiolated seedling.

REFERENCES

(1) Ewins, A. J., *J. Chem. Soc.*, **101**, 544(1912).
(2) Mitchell, H. K., and Nyc, J. F., *Proc. Natl. Acad. Sci. U.S.*, **34**, 1(1948).
(3) Keller, O., *Arch. Pharm.*, **246**, 1(1908).
(4) Keller, O., *ibid.*, **242**, 299(1904).
(5) Tunmann, O., and Rosenthaler, L., "Pflanzenmikrochemie," 2nd ed., Gebrüder Borntraeger, Berlin, 1931, p. 459.
(6) Meyer, B. S., and Anderson, D. B., "Laboratory Plant Physiology," 2nd ed., D. Van Nostrand Company, Inc., New York, 1947, p. 91.
(7) Youden, W. J., "Statistical Methods for Chemists," John Wiley & Sons, Inc., New York, 1951, p. 25.
(8) James, W. O., *J. Pharm. and Pharmacol.*, **5**, 809 (1953).

Microtoxicology XI: Identification and Differentiation of Seven New Synthetic Antispasmodics*

By THOMAS J. HALEY† and GEORGE L. KEENAN‡

The newer antispasmodic drugs, Trasentine® 6-H hydrochloride, Bentyl® hydrochloride, 2,2-diphenyl-4-dimethylaminomethyl-1,3-dioxolane hydrochloride and methobromide, 5-diethylamino-2,2-diphenylpentanenitrile hydrochloride, 2-diethylaminoethyl-cyclopentyl-hydroxy-2-thienylacetate hydrochloride have been subjected to a series of colorimetric and precipitation reagents. Definite differences in reaction of the drugs were observed, but the results were not conclusive. On the other hand, combination of melting point data and optical crystallographic measurements give positive identification of the compounds with very small samples. Such methods should be of interest to toxicologists.

RECENTLY HALEY AND KEENAN (1) published identification procedures for several of the newer synthetic antispasmodics. To obtain comparable data, the following drugs of this same group were investigated using the same procedures: Trasentine 6H hydrochloride (2-diethylaminoethyl-diphenyl-acetate HCl); Bentyl® hydrochloride (2-diethylaminoethyl-cyclohexyl-hexahydrobenzoate HCl); D-2® hydrochloride (2,2-diphenyl-4-dimethylaminomethyl-1,3-dioxolane HCl); D-2® methobromide (2,2-diphenyl-4-dimethylaminoethyl-1,3-dioxolane methobromide); Win-1550 hydrochloride (5-diethylamino-2,2-diphenylpentanenitrile HCl); Win-2299 hydrochloride (2-diethylaminoethyl-cyclopentyl-hydroxy-2-thienylacetate HCl) and Win-2760 hydrochloride (1-methyl-3-piperdylmethyl-phenyl-2-thienylacetate HCl).

EXPERIMENTAL

The uncorrected melting points of the drugs were determined with a Melt-Pointer electrically heated apparatus using capillary tubes and Anschutz thermometers.

The reagents were the same as used previously (1, 2). The compounds were tested by placing one drop of reagent on a microscope slide or in the well of a spot-plate or shallow-welled slide and adding about 0.1 mg. of drug. The results of this addition were observed for approximately thirty minutes and the changes occurring recorded. In the case of the acidic colorimetric reagents, the end point was the formation of a stable color or charring of the drug, but all colors appearing prior to this were used for identification. As a further aid in identifying and differentiating these antispasmodics, their optical crystallographic properties were studied by the immersion method after recrystallization from ethanol ether.

RESULTS

The data obtained are arranged in tabular form, melting points (Table I), colorimetric reactions (Table II), precipitation reactions (Table III), and optical crystallographic properties (Table IV).

TABLE I.—MELTING POINTS OF ANTISPASMODICS

Compound	Melting Point, °C
Trasentin 6H HCl	145–147
Bentyl HCl	165.6–166.2
D-2 HCl	194.4–195
D-2 Methobromide	216.6–217 dec.
Win-1550 HCl	154–155
Win-2299 HCl	172–172.5
Win-2760 HCl	170–170.5

* Received August 13, 1954, from the Division of Industrial Medicine, Department of Medicine, University of California at Los Angeles.
† Associate Clinical Professor of Medicine (Industrial).
‡ Formerly Microanalyst, Food and Drug Administration. Deceased.
The authors wish to thank Ciba Pharmaceutical Products, Inc. for the Trasentin 6H; Wm. S. Merrell and Company for the Bentyl and D-2® compounds and Sterling-Winthrop for the Win-compounds used in this study.

TABLE II.—COLORIMETRIC REACTIONS OF SEVEN NEW SYNTHETIC ANTISPASMODICS

Reagent	Trasentin 6H	Bentyl HCl	D-2 HCl	D-2 Methobromide	Win-1550 HCl	Win-2299 HCl	Win-2760 HCl
Conc. H_2SO_4	Colorless	Colorless	Faint yellow	Faint yellow	Faint yellow	Bright reddish orange	Yellowish brown
Conc. HNO_3	Colorless	Colorless	Colorless	Yellowish orange	Colorless	Emerald green changing to yellowish orange	Lavender to fuchsia to emerald green to dirty green to brown to red brown
Mandelin	Green	Faint red changing to green	Red	Colorless	Colorless	Colorless	Colorless
Marquis	Reddish orange to orange brown	Very light orange to orange brown	Very light green	Faint yellow to light orange	Brown	Emerald green changing to reddish purple	Reddish orange changing to reddish brown
Buckingham	Colorless	Faint yellow to faint blue	Light greenish yellow	Reddish brown to brown to emerald green to royal blue	Faint yellow to faint blue	Deep purple	Deep purple
Frohde	Colorless	Colorless	Greenish yellow	Greenish yellow to deep yellow to orange yellow	Greenish yellow	Magenta to orange brown	Orange to light brown to dark brown to purple
Vitali	Orange	Colorless	Colorless	Colorless	Colorless	Brown	Brown

TABLE III.—PRECIPITATION REACTIONS OF SEVEN NEW SYNTHETIC ANTISPASMODICS

Reagent	Trasentin 6H HCl	Bentyl HCl	D-2 HCl	D-2 Methobromide	Win-1550 HCl	Win-2299 HCl	Win-2760 HCl
Chloro-auric Acid	Oily globules. Amorphous ppt.	Amorphous ppt.	Amorphous ppt.	Reagent turns carmine red, thin needles in sheaves	Amorphous ppt.	Reagent turns reddish brown. Amorphous ppt.	Reagent turns reddish purple. Amorphous ppt.
Chloro-platinic Acid	Amorphous ppt.	Granular amorphous ppt.	Amorphous ppt.	Brown amorphous ppt.	Amorphous ppt.	Amorphous ppt.	Amorphous ppt.
Satd. Ag. Soln. Picric Acid	Amorphous ppt.	Oily globules	Needles in bundles and sheaves	Large feather-like crystals	Amorphous ppt.	Amorphous ppt.	Amorphous ppt.
Wagner	Oily globules	Long thin needles in bundles and sheaves	Flat plates and long flat rod-like forms with blunt ends	Small granules and flat knife-shaped plates	Oily globules	Oily globules	Oily globules
Mayer	Amorphous ppt.	Long thin needles in bundles and sheaves	Amorphous ppt.	Amorphous granules	Amorphous ppt.	Amorphous ppt.	Amorphous ppt.
Marme	Amorphous granules	Amorphous granules	Small needles and small flat plates	Agglomerates of small fine needles	Amorphous	Amorphous	Very small needles
Scheibler	Amorphous ppt.	Dendrites	Amorphous ppt.	Amorphous ppt.	Amorphous ppt.	Amorphous ppt.	Amorphous ppt.
Potassium Chlorate 10%	Flat plates in trapezoid forms	Amorphous granules	Tree-like forms on drying	No change	No change	Irregular flat plates, twining rods	Octahedrons
Potassium Chromate 5%	Small flat plates	Amorphous ppt.	Plates in scale-like formations	Long needles and irregular rectangular forms	Orange-colored amorphous ppt.	Orange-colored dentrites	Small crystalline granules

TABLE IV.—OPTICAL CRYSTALLOGRAPHIC PROPERTIES OF NEWER ANTISPASMODICS

Drug	Habit	Extinction	Elongation	2E	Optical sign	Refractive Indices[a] α	β	γ
Trasentine 6H HCl	Flakes	...	...	...	...	1.533	Indet.[b]	1.575
Bentyl HCl	Plates	Parallel	±	80°(±)	+	1.536	1.542	1.548
D-2 HCl	Rods	Parallel	+	40°(±)	−	1.580	1.596	1.610
D-2 Methobromide	Rods	Parallel	+	...	+	1.590	1.600	1.630
Win-1550 HCl	Fragments	...	...	...	+	1.580	1.600	1.620
Win-2299 HCl	Plates and rods	Parallel and Inclined	±	...	−	1.570	1.575	1.600
Win-2760 HCl	Flakes	...	...	...	...	1.590	Indet.[b]	1.620

[a] ±0.002.
[b] Indeterminant.

DISCUSSION

Although the colorimetric reactions are not conclusive in themselves, they do assist in differentiation of the antispasmodics from one another. Marquis reagent gave the greatest variety of color reactions of all the reagents used and the reactions differed sufficiently from those previously reported for other members of this general drug group (1) in that the colors obtained are a material aid in differentiation of the various compounds. However, care should be exercised in attempting to differentiate these compounds from one another on the basis of color reactions alone. It is better to use a combination of melting points, precipitation reactions, color reactions, and optical crystallographic properties. The latter properties offer a simple and rapid means for differentiation of these antispasmodics from each other and other drugs and employ a minimum of time and material.

SUMMARY

The melting points, reactivity to colorimetric and precipitation reagents, and the optical crystallographic properties of Trasentine® 6H Hydrochloride, Bentyl® hydrochloride, D-2® hydrochloride, D-2® methobromide, Win-1550 hydrochloride, Win-2299 hydrochloride and Win-2760 hydrochloride have been investigated. The combination of these tests enables the compounds to be differentiated from one another. Optical crystallographic procedures offer the best means for positive identification of these compounds.

REFERENCES

(1) Haley, T. J., and Keenan, G. L., THIS JOURNAL, 43, 46(1954).
(2) Thienes, C. H., and Haley, T. J., "Clinical Toxicology," 2nd ed.; Lea and Febiger, Philadelphia, Pa., 1948, pp. 298–304.

Central Depressant Properties of Uracil and Related Oxypyrimidines*

By DUANE G. WENZEL† and MORENO L. KEPLINGER

Uracil, thymine, 6-methyluracil and thiouracil were found to potentiate the mouse-hexobarbital sleeping time. The compounds were inactive against metrazol shock, but protected mice from maximal electroshock seizures. The acute toxicities of the uracils for mice were slight.

SEVERAL NATURALLY OCCURRING COMPOUNDS possess an oxypyrimidine moiety similar to that of the barbiturates. These are uracil, thymine, and alloxan. Alloxan has been found to be diabetogenic (1) while the thio derivatives of uracil are well known for their goitrogenic activity (2). The presence of uracil and thymine in nucleic acids has provided an impetus for extensive experimentation as to their function in bacterial metabolism. It was observed that barbituric acid inhibited certain uracil-dependent bacteria (3) as well as some requiring thymine (4). This antagonism placed barbituric acid in the role of an antimetabolite. Neither Wolf (5) nor Sweet and Laverne (6) could find any pharmacological actions of uracil or thymine. It has been shown that uracil and thymine are broken down by both the liver and kidneys but that 6-methyluracil is only partially broken down by these organs (7–10).

Although the primary purpose of this problem was to determine if the bacterial antagonism of these compounds would extend to the hypnotic effect of the barbiturates upon the higher or-

* Received August 7, 1954 from University of Kansas, School of Pharmacy, Lawrence. Abstracted from a portion of the data contained in a thesis submitted to the Graduate School of the University of Kansas by Moreno L. Keplinger for the degree of Master of Science.
† Associate Professor of Pharmacology, School of Pharmacy, University of Kansas, Lawrence.

ganism, the initial results extended the scope of the study to include barbiturate potentiation and anticonvulsant testing.

EXPERIMENTAL

Materials.—Uracil, thymine, thiouracil, 6-methyluracil and sodium hexobarbital were obtained from commercial sources. Because the oxypyrimidines are almost completely water-insoluble the sodium salts were prepared for parenteral administration. Sodium thymine was obtained by dissolving equimolar quantities of thymine and sodium hydroxide in absolute alcohol and refluxing for four hours. The salt crystallized from the solution on cooling and was recrystallized from 95% alcohol. A satisfactory product was not obtained using this method with the other compounds. Uracil, 6-methyluracil and thiouracil were dissolved in equimolar quantities of a 4% aqueous solution of sodium hydroxide and the water removed in a vacuum desiccator. The *p*H's of the sodium uracils in the solutions to be injected ranged from 12.10 to 13.00 but by using a sodium biphosphate buffer the *p*H's were reduced to the range of 7.62 to 9.48. Male albino mice weighing from 20 to 25 grams were used for all tests.

Hexobarbital Sleeping Time.—Doses of the uracils up to 1,500 mg./Kg. given orally as a suspension or intraperitoneally as solutions of the sodium salts produced no apparent hypnotic effect upon mice. The drugs were then tested for their action upon the mouse-hexobarbital sleeping time. Six groups of 10 mice per group were used. One control group was administered 100 mg./Kg. of sodium hexobarbital intraperitoneally. A second control group was given a buffered saline solution i.p. at a dosage level of 120 mg./Kg. and *p*H of 9.6 so as to correspond to the highest dose and *p*H level of the test drugs. After a five-minute interval this second group was injected with the 100 mg./Kg. dose of sodium hexobarbital. The 4 remaining groups were respectively administered 1 mM/Kg. of the sodium salts of uracil, 6-methyluracil, thiouracil and thymine and after five minutes injected with 100 mg./Kg. of sodium hexobarbital.

The oral activity of the uracils was tested similarly with doses of 2 mM/Kg. administered as suspensions and followed in thirty minutes by the i.p. injection of 100 mg./Kg. of sodium hexobarbital. The control group in this instance was administered 250 mg./Kg. of lactose in lieu of an oxypyrimidine. The mice were fasted for eighteen hours prior to the administration of the drugs.

Sleeping time was measured to the nearest second with a stopwatch from the time of injection of sodium hexobarbital until the mouse became sufficiently coordinated to climb out of a metal box $4^1/_2$ in. x $2^1/_2$ in. x $1^1/_2$ in. equipped with an air jet at each lower corner pointing at the opposite upper corner of the box. The air was passed through a graphite lubricated stopcock actuated by a variable speed motor. The speed of the motor was arbitrarily selected to deliver approximately 90 blasts of air per minute through the box.

Metrazol® Seizures.—The uracils were tested at doses of 6 and 12 mM/Kg. Administration was peroral to groups of 6 mice followed in forty minutes by the intraperitoneal injection of 100 mg./Kg. of Metrazol.® This amount of Metrazol® has been shown to be 97% of the convulsive dose for mice (11). An animal was considered to be protected if clonic convulsions persisting for more than five seconds were prevented (12).

Maximal Electroshock Seizures.—A modification of the method of Toman, *et al.* (12), was employed to produce convulsions in mice. Prior to testing, the animals were fasted for eighteen hours. Stimuli were delivered at periods of thirty, ninety, one hundred and fifty, two hundred and ten, and two hundred and seventy minutes following the oral administration of the uracil. It has been demonstrated that hour intervals between stimuli provide sufficient time to restore approximately normal seizure thresholds (13). A current of 24 ma, which is 3–5 times the threshold convulsive level, was applied for two-tenths second through ear electrodes. The criterion of protection was inhibition of the tonic extensor phase of the convulsions. Eight mice were used per dose and from 5–6 doses tested for each drug. A geometric progression of 1.5 was used between doses. Results were graphically estimated by the method of Litchfield and Wilcoxon (14) to obtain the PD_{50} in terms of millimoles for each drug at each of the times tested.

Acute Toxicity.—Toxicity of the compounds was tested intraperitoneally as the sodium salts and orally as suspensions with groups of 8 mice per dose. Five dose levels of each drug arranged in a geometric progression were used. The LD_{50} were calculated in the same manner as the PD_{50} of the maximal electroshock seizures (14).

RESULTS AND DISCUSSION

Although devoid of hypnotic activity, it is apparent from the data in Table I that the uracils tested increase the mouse-hexobarbital sleeping time. Uracil exhibited the most pronounced potentiation. Intraperitoneally the sleeping time increase produced by 1 mM/Kg. was almost 60% and orally, 2 mM/Kg. caused a 78% increase. 6-Methyluracil was next in order of activity while thiouracil and thymine varied according to the route of administration.

TABLE I.—EFFECT OF URACILS ON MOUSE-HEXOBARBITAL[a] SLEEPING TIME

Compounds	Sleeping Time in Seconds ± S.E.[b] Intraperitoneal, 1 mM/Kg.	Oral, 2 mM/Kg.
Control	1,703 ± 104	1,703 ± 104
Saline *p*H 9.6	1,771 ± 103	.....
Lactose	.....	1,689 ± 29
Uracil	2,711 ± 139	3,023 ± 65
Thymine	2,323 ± 141	2,250 ± 84
6-Methyluracil	2,502 ± 147	2,943 ± 92
Thiouracil	2,018 ± 85	2,530 ± 45

[a] 100 mg./Kg. intraperitoneally.

[b] S.E. = $\sqrt{(\Sigma Y^2 - \bar{Y}T)/[N(N-1)]}$.

Many compounds have been observed to increase the depressant effects of the barbiturates. Given orally, iodides and bromides (15), tetraethylthiuramdisulfide (16–20) and β-diethylaminoethyldiphenylpropyl acetate HCl (SKF 525A) (21–23) prolong the length of barbiturate hypnosis. The intraperitoneal or intravenous administration of

TABLE II.—MAXIMAL ELECTROSHOCK PROTECTION[a]

Compound	Time in Minutes 30	90	150	210	270
Uracil	2.23 ± 0.08	3.35 ± 0.10	6.69 ± 0.28	8.93 ± 0.39	.....
Thymine	1.86 ± 0.23	3.17 ± 0.15	4.76 ± 0.23	7.15 ± 0.34	8.73 ± 0.39
6-Methyluracil	2.62 ± 0.12	3.17 ± 0.13	6.19 ± 0.26	34.33 ± 0.40	.....
Thiouracil	1.72 ± 0.09	3.32 ± 0.17	.....	.....	.....

[a] PD_{50}'s expressed as mM/Kg.

cholesterol (24, 25), antihistaminics (26), thiamine (27), nitrites (28) and glucose or its metabolic products (29–31) will reinstate, intensify, or prolong the hypnosis. The mechanisms by which these agents act undoubtedly differ widely. The action of the uracils apparently does not resemble the epinephrine-mediated "glucose effect" as glucose is relatively ineffective for prolonging hypnosis, has no action upon mice and is inactive orally (30). It is the opinion of Krantz and Fassel (15) that the injection of agents having a high osmotic pressure causes a redistribution of body fluid and thereby affects the brain barbiturate concentration. The activity of the uracils cannot be explained in this manner because of their effectiveness via the peroral route. There is also no reason to believe that they act as do the nitrites by increasing the cerebral blood supply through vasodilatation (28).

Enzyme inhibition has been stated to be the method by which certain barbiturate-potentiating drugs are active. SKF 525A is believed to potentiate barbiturates by blocking sidechain oxidation (21, 22) while the tetraethylthiuramdisulfide increase of barbiturate sleeping time has been attributed to xanthine oxidase inhibition (17), aldehyde dehydrogenase inhibition (18) as well as a direct additive depression (19). The close structural similarity of the barbiturates and uracils and their antagonism for microorganisms (1, 2) raises the possibility of competition for enzymes involved in the biotransformation of hexobarbital. The fact that the uracils (4–8) and hexobarbital (32, 33) are destroyed by liver may indicate this organ as a potential site of competitive biotransformation. 6-Methyluracil, however, is only partially broken down by the liver or kidneys (4–8) but is very active in prolonging the sleeping time. Further study is required to establish the mode of action.

No detectable degree of protection was exerted against Metrazol® convulsions at either of the dosage levels tested, but all compounds demonstrated electroshock anticonvulsant activity. Peak protection was observed at thirty minutes and, as shown in Fig. 1 and Table II, activity had dropped below the PD_{50} at four and one-half hours for all compounds except thymine.

The anticonvulsant activity may obviously be due to a direct effect of the uracils on the central nervous system, but another mechanism is possible. Uracil has been demonstrated to exert a slight thyroid inhibition (2) and 6-methyluracil has controversial antithyroid activity (34, 35). 6-Propylthiouracil, a potent antithyroid agent, decreases the brain excitability to both metrazol and electroshock (36) and audiogenic seizures (37). While thyroidectomy was also found to protect against convulsions, certain of the effects of 6-propyl thiouracil were different. This was attributed to a direct central depressant effect of the 6-propylthiouracil (36).

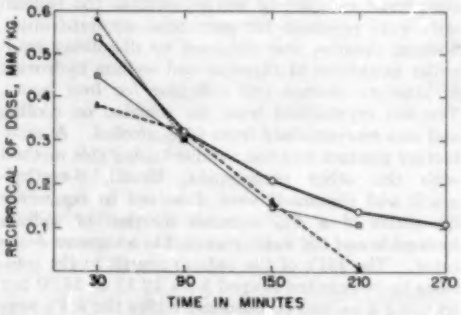


Fig. 1.—Duration of protection of uracils for mice against maximal electroshock. ●, thiouracil; Δ, 6-methyl uracil; □, uracil; ○, thymine.

Although it is conceivable that the uracils induce their anticonvulsant activity through both antithyroid and a direct central depressant action, it would appear that the role of the thyroid is a minor one. This is borne out by the lack of relationship between anticonvulsant activities as tested and the reported antithyroid values of the uracils (34–36).

As indicated by Table III the LD_{50} of 6-methyluracil and uracil were not determinable because at doses higher than 7500 mg./Kg. the solution volume or viscosity became too great.

TABLE III.—ACUTE TOXICITY

Compound	LD_{50}, mg./Kg.[a]
Uracil	>7,500
Thymine	3,500 (2966–4130)
6-Methyluracil	>7,500
Thiouracil	3,900 (3120–4875)

[a] Figures in parentheses are 95% confidence limits.

SUMMARY

1. Uracil, thymine, thiouracil and 6-methyluracil were found to be devoid of apparent central depressant action *per se* but prolonged the mouse-hexobarbital sleeping time.

2. The compounds were ineffective against Metrazol® convulsions of mice but were active in preventing maximal electroshock seizures.

3. A low order of oral toxicity was evidenced for mice.

REFERENCES

(1) Dunn, J. S., Sheehan, H. L., and McLetchie, N. G. B., *Lancet*, **1**, 484(1943).
(2) Astwood, E. B., *J. Pharmacol. Exptl. Therap.*, **78**, 79(1943).

(3) Woods, D. D., *Nature*, **148**, 758(1941).
(4) Hitchings, G. H., Falco, E. A., and Sherwood, M. B., *Science*, **102**, 251(1945).
(5) Wolf, C. H., *J. Physiol.*, **32**, 171(1905).
(6) Sweet, R. D., and Laverne, J. C., *J. Exptl. Med.*, **9**, 229(1907).
(7) Mendel, L. B., and Myers, V. C., *Am. J. Physiol.*, **26**, 77(1910).
(8) Kleiner, I. S., *J. Biol. Chem.*, **11**, 443(1912).
(9) Cerecedo, L. R., *ibid.*, **75**, 661(1927).
(10) Stekol, J. A., and Cerecedo, L. R., *ibid.*, **100**, 653(1933).
(11) Berger, F. M., *Proc. Soc. Exptl. Biol. Med.*, **71**, 270 (1949).
(12) Toman, J. E. P., Swinyard, E. A., and Goodman, L. S., *J. Neurophysiol.*, **9**, 231(1946).
(13) Toman, J. E. P., Swinyard, E. A., Merkin, M., and Goodman, L. S., *J. Neuropathol. Exptl. Neurol.*, **7**, 35(1948).
(14) Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharmacol. Exptl. Therap.*, **96**, 99(1948).
(15) Krantz, J. C., Jr., and Fassel, M. J., THIS JOURNAL, **40**, 511(1951).
(16) Johnson, C. D., Harkness, W. D., and Woodard, G., *Fed. Proc.*, **12**, 333(1953).
(17) Giarman, N. J., Flick, P. H., and White, A. M., *Science*, **114**, 35(1951).
(18) Graham, W. D., Carmichael, E., and Allmark, M. G., *J. Pharm. Pharmacol.*, **3**, 497(1951).
(19) Winters, W. D., and Shideman, F. E., *Fed. Proc.*, **13**, 417(1954).
(20) Graham, W. D., Teed, H., and Allmark, M. G., *J. Pharm. Pharmacol.*, **4**, 615(1952).
(21) Axelrod, J., Reichenthal, J., Quinn, G. P., and Brodie, B. B., *Fed. Proc.*, **12**, 299(1953).
(22) Cooper, J. R., and Brodie, B. B., *ibid.*, **12**, 313 (1953).
(23) Cook, L., Toner, J. J., and Fellows, E. J., *J. Pharmacol. Exptl. Therap.*, **111**, 131(1954).
(24) Starkenstein, E., and Weden, H., *Arch. exptl. Pathol. Pharmakol.*, **182**, 700(1936).
(25) Farson, de C. B., Carr, C. J., and Krantz, J. C., Jr., *J. Pharmacol. Exptl. Therap.*, **89**, 222(1947).
(26) Winter, C. A., *ibid.*, **94**, 7(1948).
(27) De Boer, B., THIS JOURNAL, **37**, 302(1948).
(28) Wooster, H. A., Jr., and Sunderman, F. W., *J. Pharmacol. Exptl. Therap.*, **97**, 140(1949).
(29) Lamson, P. D., Greig, M. E., and Robbins, B. H., *Science*, **110**, 690(1949).
(30) Lamson, P. D., Greig, M. E., and Hobdy, C. J., *J. Pharmacol. Exptl. Therap.*, **103**, 460(1951).
(31) Lamson, P. D., Greig, M. E., and Williams, L., *ibid.*, **106**, 219(1952).
(32) Werner, H. W., Pratt, T. W., and Tatum, A. L., *ibid.*, **60**, 189(1937).
(33) Das, S. C., and Raventós, J., *Quart. J. Exptl. Physiol.*, **29**, 343(1939).
(34) Jensen, K. A., and Kjerulf-Jensen, K., *Acta Pharmacol. Toxicol.*, **1**, 280(1945).
(35) Freiesleben, E., Kjerulf-Jensen, K., and Schmith, K., *ibid.*, **1**, 82(1945).
(36) Woodbury, D. M., Hurley, R. E., Lewis, N. G., Kirschvink, J. F., and Goodman, L. S., *J. Pharmacol. Exptl. Therap.*, **106**, 331(1952).
(37) Vicari, E. M., *Proc. Soc. Exptl. Biol. Med.*, **78**, 744 (1951).

Anti-Metrazol® Efficacy of Combinations of Meparfynol and Anticonvulsant Drugs*

By DAVID A. BRODIE,† ARTHUR TYE,‡ and JOHN W. NELSON§

Combinations of meparfynol and anticonvulsant drugs were tested for anti-Metrazol® potency and neurotoxicity in mice. The addition of meparfynol to an anticonvulsant did not cause the protective index to rise above that of the better component in the combination.

MEPARFYNOL[1] (Dormison, methylparafynol, 3-methylpentyne-3-ol) was originally introduced as a hypnotic (1). It was later reported by Schaffarzick and Brown (2) to possess anticonvulsant properties and to potentiate the anticonvulsant action of phenobarbital. A subsequent study (3) verified the anticonvulsant activity of this compound and that of some other unsaturated carbinols and indicated that certain of these drugs deserved cautious clinical trial in epilepsy. In view of its reported ability to potentiate phenobarbital and because meparfynol represents a class of chemicals entirely unrelated to the conventional antiepileptic ring structures, it was thought important to see if the margins of safety of drug combinations composed of meparfynol and a clinically useful anticonvulsant were improved over those of the individual drugs forming the combination. Accordingly, the anti-Metrazol® potency and neurotoxicity of combinations of meparfynol and phenacemide, meparfynol and phenobarbital, and meparfynol and trimethadione were determined and the protective indices calculated. For comparative purposes a combinationm of eparfynol and paraldehyde was included also. The results obtained provide the basis for this report.

EXPERIMENTAL

Methods.—Adult white mice of the Wistar strain weighing from 14 to 30 Gm. were used as experimental animals. They were maintained on an adequate diet and allowed free access to food and water except during the period of testing. All drugs were used in aqueous solution with the exception of phenacemide, which was suspended in 5% acacia mucilage. All drugs were administered intraperitoneally and their concentrations so adjusted that the volume injected would not exceed 1 cc.

The anti-Metrazol® potency of the individual drugs and drug combinations was measured by ability to protect test animals against the lethal dose (LD_{99}) of Metrazol® (pentylenetetrazol) which was determined to be 120 mg./Kg.; this method has been reported previously (4, 5). The Metrazol® was injected subcutaneously at the time of peak effect of each drug. The death of the animal within a period of ninety minutes after the Metrazol® injection was considered

* Received March 4, 1954 from the College of Pharmacy, The Ohio State University, Columbus.
† Present address: School of Medicine, University of Utah, Salt Lake City.
‡ Associate Professor, College of Pharmacy, The Ohio State University.
§ Professor, College of Pharmacy, The Ohio State University.
[1] Supplied by the Schering Corporation, Bloomfield, N. J.

TABLE I.—ANTI-METRAZOL® POTENCY, TOXICITY, AND PROTECTIVE INDICES OF MEPARFYNOL AND COMBINATIONS

Drug	Combination	Potency (PD_{50}), mg./Kg.	Neurotoxicity (TD_{50}), mg./Kg.	Protective Index (P.I.), TD_{50}/PD_{50}
Meparfynol	.....	65.0 (59.6–70.9)[a]	160 (156–164)	2.46 (2.24–2.71)
Paraldehyde	.....	235.0 (213.0–248.0)	468 (453–485)	1.99 (1.81–2.09)
	Meparfynol, 26% / Paraldehyde, 74% \	170.0 (155.0–198.0)	368 (350–384)	2.16 (1.83–2.55)
Phenacemide	.....	99.0 (89.0–110.0)	312 (286–340)	3.17 (2.83–3.55)
	Meparfynol, 34% / Phenacemide, 66% \	99.3 (81.3–122.0)	298 (285–311)	3.01 (2.41–3.74)
Phenobarbital	.....	19.0 (15.9–22.8)	106 (103–110)	5.58 (4.68–6.69)
	Meparfynol, 60% / Phenobarbital, 40% \	39.0 (31.9–47.1)	142 (135–149)	3.64 (2.93–4.52)
Trimethadione	.....	340.0 (322.0–358.0)	900 (873–927)	2.64 (2.40–2.90)
	Meparfynol, 15% / Trimethadione, 85% \	230.0 (214.0–246.0)	599 (533–565)	2.43 (2.20–2.67)

[a] 95% confidence limits.

as failure to protect (animals which survived the ninety-minute period invariably lived).

Dose-effect curves were established for the neurotoxicity and anti-Metrazol® potency of each drug and drug combination using at least 3 groups of 12 animals at 3 dose levels. Minimum neurological toxicity was measured by the method suggested by Swinyard, *et al.* (6). Failure of the animal to walk a narrow cage rim and maintain its equilibrium was interpreted as evidence of neurological deficit. Doses of each drug and drug combination that produce toxicity in 50% of the animals (TD_{50}) and that protect 50% of the animals (PD_{50}) were determined by the graphic method of Litchfield and Wilcoxon (7), and their protective indices (TD_{50}/PD_{50}) calculated. The percentages of the components employed in the combinations were chosen after preliminary tests for neurotoxicity; combinations employed were those which exhibited the least toxicity.

RESULTS

A comparison of the protective indices (P.I.'s) of the drugs (Table I) shows that phenobarbital has the widest margin of safety, followed by phenacemide, trimethadione, meparfynol and paraldehyde. An examination of the P.I.'s of the drug combinations, as compared to the component drugs, reveals that meparfynol with paraldehyde, meparfynol with phenacemide, and meparfynol with phenobarbital have P.I.'s intermediate to those of the component drugs in the mixture. The P.I. of the combination of meparfynol with trimethadione was below that of both components. Thus, the combination of meparfynol with the anticonvulsant drugs caused no striking alteration in the protective index of any combination over that of the individual components in the mixture.

DISCUSSION

The potency ranking of the clinically useful anticonvulsants (Table I) is the same as that reported by Swinyard, *et al.* (6), using a Convulsive Dose 97 of Metrazol® (85 mg./Kg.); namely, phenobarbital is most potent, with phenacemide and trimethadione following in order. Their neurotoxicity ranking (Table I) follows the same order as the anti-Metrazol® potency. This agrees with the work previously published (6).

A definitive analysis of drug combinations may be made by the method suggested by Finney (8). However, there are many problems attending such studies. Because the drugs selected for this investigation represent different chemical nuclei, and since they are not all of the same therapeutic class, it was not considered prudent to predict the efficacy of these drug combinations on the basis of the intensity of their anti-Metrazol® and neurotoxicity effects. Furthermore, Loewe (9) has called attention to the fact that such terms as synergism, antagonism, and analogous terms for supra- and infra-additive effects of combined drugs are indefinable. In addition, he has indicated that it is much more important to compare the ratios between the intensities of effects, i. e., the margins of safety or protective indices, than it is to compare the intensities of various effects of the combinations themselves. Therefore, it was thought best to consider a combination better than its components if its P.I. was markedly greater than the P.I.'s of the individual drugs.

A comparison of the P.I.'s of the combinations and component drugs shows that the rank of the combinations is the same as that of the component drugs. The best combination is that of meparfynol and phenobarbital, followed in order by meparfynol with phenacemide, meparfynol with trimethadione, and meparfynol with paraldehyde. Thus, the addition of meparfynol to the anticonvulsants did not change the relationships of the P.I.'s from that of the component drugs themselves, and did not increase the protective index over that of the better component in the combinations.

SUMMARY AND CONCLUSIONS

1. The anti-Metrazol® activity and neurotoxicity were determined in mice for meparfynol, paraldehyde, phenobarbital, phenacemide, and trimethadione, and for combinations of meparfynol with these drugs.

2. The order of the protective indices of the drugs from highest to lowest was found to be phenobarbital, phenacemide, trimethadione, meparfynol and paraldehyde.

3. The order of protective indices of the combinations from highest to lowest was found to be

meparfynol with phenobarbital, meparfynol with phenacemide, meparfynol with trimethadione and meparfynol with paraldehyde.

4. Combinations of meparfynol with phenobarbital, meparfynol with phenacemide, meparfynol with trimethadione, and meparfynol with paraldehyde exhibited protective indices between or below those of the component drugs.

REFERENCES

(1) Margolin, S., Perlman, P. L., Villani, F. J., and McGavack, T. H., *Science*, **114**, 384(1951).
(2) Schaffarzick, R. W., and Brown, B. J., *Stanford Med. Bull.*, **10**, 106(1952).
(3) Swinyard, E. A., Schiffman, D. O., Brown, W. C., Grewal, M. S., and Goodman, L. S., *Arch. Intern. Pharmacodynamie*, **94**, 81(1953).
(4) Berger, F. M., *Proc. Soc. Exptl. Biol. Med.*, **71**, 270 (1949).
(5) Everett, G. M., and Richards, R. H., *J. Pharmacol. Exptl. Therap.*, **81**, 402(1944).
(6) Swinyard, E. A., Brown, W. C., and Goodman, L. S., *ibid.*, **106**, 319(1952).
(7) Litchfield, J. T., and Wilcoxon, F., *ibid.*, **106**, 303 (1949).
(8) Finney, D. J., "*Probit Analysis*," 2nd ed., Cambridge University Press, London, 1952, p. 130.
(9) Loewe, S., *Arzneimittel-Forsch.*, **3**, 285(1953).

A Note on the Use of Amberlite® IRC-50 for the Purification of Nonaqueous Solvents*

By LEO LEVI, LESLIE G. CHATTEN, and M. PERNAROWSKI

Most solvents employed in nonaqueous titrations are, as a rule, readily available from suppliers in grades which require no further purification before use. Two notable exceptions are acetonitrile and dioxane.

Acetonitrile.—This solvent medium has been successfully used by Fritz (1), Fritz and Keen (2), Freeman (3) and others for the determination of a number of nitrogenous bases. Unfortunately, because of the presence of basic impurities in the reagent, a relatively large correction factor must frequently be applied in these titrations. Recent investigations with various samples of acetonitrile showed that in some instances as much as 0.50 ml. of 0.05 *N* perchloric acid in dioxane was required to neutralize 50 ml. of the solvent (Fig. 1). Blanks of this size are a most undesirable feature in accurate analytical work because they indicate that sufficient impurities may be present to cause interfering side reactions. The authors found that these blanks could be reduced to approximately 0.01 to 0.02 ml. following treatment of the solvent with Amberlite® IRC-50. This volume of titrant compared favorably with that for a similar sample of spectrally pure acetonitrile. Purification was carried out by shaking intermittently over a twenty-four hour period one liter of acetonitrile with 75–100 Gm. of resin and filtering through Whatman No. 42 filter paper. No deterioration of the purified material was observed during the six-week period of this investigation.

It may be of interest to note that the refractive index of the solvent remained unchanged by this purification process. Refractivity measurements are therefore not to be considered a reliable indication of the quality of this reagent for nonaqueous titrimetry.

Dioxane.—This solvent is widely used for the preparation of perchloric acid solutions employed as titrants in nonaqueous media. Frequently, however, such solutions discolor on standing because of the presence of solvent impurities. According to Sideri and Osol (4), dioxane may be purified by shaking with asbestos for an hour or so. We were not able to apply this method with equal success—probably due to the use of a different grade of asbestos. Pifer, Wollish, and Schmall (5) purified dioxane by treatment with Amberlite® IRA-400 and reported satisfactory results. The present authors purified various samples of low grade dioxane utilizing Amberlite® IRC-50 as described above and observed test solutions of 0.05 *N* perchloric acid in both purified and unpurified solvent. While the latter turned yellowish brown within a few days, no discoloration was noted after four weeks' standing of solutions prepared from the purified solvent.

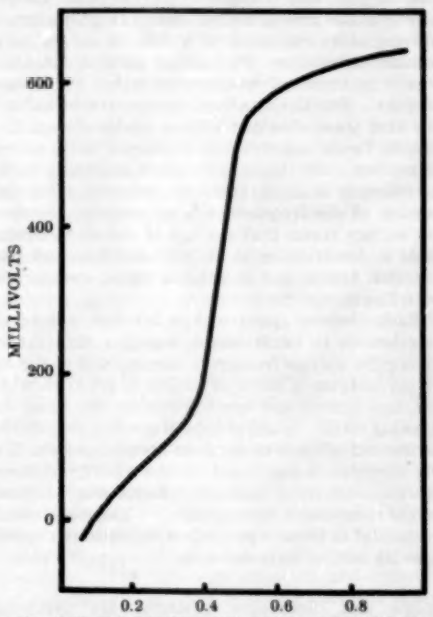


Fig. 1.—Titration of basic impurities in 50 ml. of Acetonitrile.

REFERENCES

(1) Fritz, J. S., *Anal. Chem.*, **25**, 407(1953).
(2) Fritz, J. S., and Keen, R. T., *ibid.*, **25**, 179(1953).
(3) Freeman, S. K., *ibid.*, **25**, 1750(1953).
(4) Sideri, C. N., and Osol, A., THIS JOURNAL, **42**, 586 (1953).
(5) Pifer, C. W., Wollish, E. G., and Schmall, M., *ibid.*, **42**, 509(1953).

* Received June 26, 1954, from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada.

Book Notices

The Infra-Red Spectra of Complex Molecules. By L. J. BELLAMY. John Wiley & Sons, Inc., New York, 1954. xvii + 323 pp. 22.5 x 14 cm. Price $7.

The author has limited the subject-matter of this book strictly to the empirical interpretation of infra-red spectra, and he has not attempted to cover the related aspects of practical spectroscopy such as sample preparation, cell construction, quantitative analysis, instrumentation, etc. The types of linkage affecting the spectra are roughly grouped as: I. Carbon-carbon and carbon-hydrogen. II. Carbon-oxygen and oxygen-hydrogen. III. Carbon-nitrogen and nitrogen-hydrogen. IV. Linkages involving other elements, or which are related to inorganic structures. The author expresses measurements in terms of frequencies rather than wavelengths. Practically all of the spectra included in the text were obtained with a Perkin-Elmer 21 B double beam spectrometer equipped with a rock-salt prism. For those who use instruments with a wavelength scale, a table of reciprocals for conversion of the frequencies is appended. However, the author states that the use of the wave-number scale makes it easier to identify overtone and combination bands and it enables direct comparisons with Raman spectra.

Each chapter opens with a brief outline of the correlations to be discussed, together with a table giving the various frequency ranges; and at the end of the chapter a series of charts is given in which the correlations are summarized in the usual line drawing form. Small groups of spectra are included at the end of each of the four divisions of the text, the divisions being based on the above-mentioned vibration-affecting linkages. Indexes of subjects and of compounds are appended. This book should be helpful to those who are interested in the rapidly growing field of infra-red spectroscopy.

Micro and Semimicro Methods. By NICHOLAS CHERONIS. Interscience Publishers, Inc., New York, 1954. xxiii + 628 pp. 23.5 x 16.5 cm. Price $12.

This is the sixth in a planned series of nine volumes on the Technique of Organic Chemistry. This text does not discuss theoretical considerations of micromethods at any length, because the basis for these procedures is identical with that of the macromethods presented in the earlier volumes in the series. Where the basis for a micromethod differs from that of the macroprocedure, it is developed more fully. Descriptions of applied microtechniques are given in detail. This is essential for reproducible results, particularly since the number of organic chemists who have practical experience with these procedures is relatively small.

The merits and limitations of the various tools and apparatus designed to carry out microprocedures are reviewed and discussed. While only one or two types of apparatus for a particular application are discussed in detail, their selection was based on their suitability, precision and accuracy, cost and availability, and ease of repair and replacement of parts. Illustrations of many of the described pieces of apparatus are included.

Techniques for micropreparative methods, for microsyntheses (including procedures with tracer elements), and microanalytical procedures for identification of compounds and for quantitative determination of organic compounds and their functional groups are included. The excellent presentation, the comprehensive bibliographic references, and the index make this volume a splendid addition to this valuable series. This sixth volume also contains abbreviated cumulative author and subject indexes for volumes I to VIII inclusive.

Bentley's Textbook of Pharmaceutics. 6th ed. Revised by HAROLD DAVIS, with the collaboration of M. W. PARTRIDGE, and C. L. SARGENT, with contributions by W. A. Broom, H. A. Turner, and M. Turner. Bailiere, Tindall and Cox, London. xiii + 1078 pp. 22.5 x 14.5 cm. Price $10.

The sixth edition of this textbook includes revisions based upon the latest British Pharmacopoeia (1953) and the changes in the syllabus of the examinations qualifying for pharmaceutical chemist and for registration as a pharmacist in Great Britain. The text is therefore quite comprehensive in its fields of coverage; although the extent to which the divisions in each field are presented are governed by their relative importance. Subjects such as chromatography and ion exchange are summarized and related to pharmaceutical practices in manufacture as well as to analytical methods. Manufacturing methods and equipment are treated thoroughly. Physical and chemical methods of sterilization are discussed at length. The U. S. Food and Drug Administration statement of policy (January, 1953) on sterility of ophthalmic solutions is cited, and materials incompatible with the commonly used (in Great Britain) bacteriostatic agents (chlorocresol and phenylmercuric nitrate) are listed.

This edition of Bentley's Textbook of Pharmaceutics is printed on excellent paper and includes many illustrations. It is well documented for further reference reading, and it should be available to pharmacists everywhere.

Practical Physiological Chemistry. By PHILIP B. HAWK, BERNARD L. OSER, and WILLIAM H. SUMMERSON. The Blakiston Co., Inc. New York, 1954. 13th ed. xvi + 1439 pp. 23.5 x 16 cm. Price $12.

This is the thirteenth edition of a widely-used textbook with which one of the authors, P. B. Hawk, has been actively associated for fifty years. The splendid reputation of this authoritative book will be continued in its recent revision, although even the best text must, in these days, "run a losing race with the periodical literature." In this edition the chapters dealing with nucleic acids and nucleoproteins, milk, muscular tissue, enzymes and their action, and

isotopes have been exhaustively rewritten. And in all the text an endeavor has been made to include current advances in the many fields of biochemistry which are benefiting from the utilization of newer techniques and instrumentation.

The student, the teacher, the investigator, and the clinician will find much information well presented and conveniently available in this book. However, the excellent index would be even better if the type were a little larger. Those who have used the earlier editions of this book will be glad to learn of the availability of a recent revision of the text, and the book should continue to be useful to workers in many fields of chemistry. No pharmacy library should be without a copy of this excellent text and reference book.

The Vitamins—Chemistry, Physiology, Pathology. Vol. II. W. H. SEBRELL, JR., and ROBERT S. HARRIS, Editors. Academic Press, Inc., New York, 1954. xiii + 766 pp. 9.25 x 6 cm. Price $16.50.

The second volume of this series of comprehensive monographs by authoritative contributors includes chapters on: Choline, Vitamin D Group, Essential Fatty Acids, Inositols, Vitamin K Group, Niacin, and Pantothenic Acid. The chapters follow a general outline which correlate the discussions by different contributors under the headings. Chemistry, Industrial Preparation, Biochemical Systems, Specificity of Action, Biogenesis, Estimation (physical, chemical, biological, and microbiological methods), Standardization of Activity, Occurrence, Effects of Deficiency, Pharmacology, and Requirements and Factors Influencing Them. The illustrated text material is well presented with an extensive bibliography recorded on the pages which include the respective references. Author and subject indexes are appended. This series will be a necessary addition to any complete reference library on the vitamins.

Chemical Specificity in Biological Interactions. Edited by F. R. N. GURD. Academic Press, Inc., New York, 1954. xv + 234 pp. 23 x 16 cm. Price $6.

This third volume in a series of "Memoirs" or symposia, published by the University Laboratory of Physical Chemistry Related to Medicine and Public Health, Harvard University deals with certain selected aspects of the chemical specificity underlying biological interactions. The subjects discussed by the respective authors are divided into the following chapters: Chemical Specificity in Biological Interactions (Proteins), E. J. Cohn; Effects of X-ray and Other Radiation on Proteins and Living Tissues, S. Warren; Properties of the Steroids Related to Protein Binding, R. B. Turner; Biochemical Problems of Steroid Hormones, T. F. Gallagher; Studies on the Active Principles of the Posterior Pituitary Gland, V. du Vigneaud; Influence of Ion-exchange Chromatography upon Our Concept of the Structure of Ribonucleic Acid, W. E. Cohn; Special Problems in the Formation of Metal Complexes, C. D. Coryell; Interactions of Metals with Small Molecules in Relation to Metal-Protein Complexes, J. Schubert; Specificity of Metal Complex Formation, G. Schwarzenbach; Interaction of Proteins with Small Molecules and Ions, G. Scatchard, W. L. Hughes, Jr., F. N. Gurd, and P. E. Wilcox. References are given at the end of each chapter and author and subject indexes are appended. The value of the book as an up-to-date reference volume is evident from the list of contributors.

Hydrometers and Hydrometry. By E. R. CRANDALL. Published by the author, 10,335 Elmira, Detroit 4. iii + 94 pp. 27.5 x 21.3 cm. Price $2.50.

Since the seventeenth century, hydrometers have been produced in a great variety of designs for many specialized purposes, but all apply the same physical principle and are used in similar manners. Failure by the author to locate a special treatise on the subject of hydrometers and hydrometry prompted him to compile the information covered in this well-written, mimeographed book.

The assembled material is designed to meet the need of students, teachers, and practical operators. The subject has received rather broad treatment and includes the designs of hydrometers, the physical principles involved in their use, explanations of the sources and magnitudes of error, and descriptions of many scales, both rational and arbitrary. A useful feature includes methods of converting between specific gravity and certain arbitrary scale readings of hydrometers currently in use, and those previously employed. The book also includes a list of currently used hydrometers, commercially available in the United States.

Professor Crandall, of Wayne University in Detroit, deserves commendation for having brought together information on hydrometers and hydrometry, heretofore widely scattered in the scientific literature relating to these subjects.

Recent Progress in Hormone Research. Vol. 10. G. PINCUS, Ed. Academic Press Inc., New York, 1954. 511 pp. 23.5 x 16 cm. Price $9.80.

This book constitutes the proceedings of the tenth annual meeting of the Laurentian Hormone Conference, which was held at Mt. Tremblant, Quebec in September, 1953. It includes fourteen formal presentations of papers presented at that time, each of which is followed by an abstract of the ensuing discussions. The fourteen chapters are logically arranged into six sections, covering hormone interrelationships of the nervous system, thyroid hormone physiology and biochemistry, comparative endocrinology, protein hormones, the role of hormones in blood and blood-forming organs, and aspects of clinical endocrinology.

The book is provided with an author index and a satisfactory subject index. It very adequately covers recent advances in hormone research.

Organic Analysis. Vol. 2. Editors: JOHN MITCHELL, JR., I. M. KOLTHOFF, E. S. PROSKAUER, and A. WEISSBERGER. Interscience Publishers, Inc., New York, 1954. vii + 372 pp. 16 x 23.5 cm. Price $8.50.

This is the second volume of a series designed for the use of chemists in academic institutions and in industry, covering critical information on methods for the direct analysis of organic substances. It emphasizes modern trends in organic analysis toward functional group determination by chemical and instrumental techniques.

The first volume was devoted to the determination of functional groups and other techniques used in organic analysis, and was described in THIS JOURNAL **42**, 706(1953). The present volume continues with t treatment of the microdetermination of carboxyl groups (neutralization equivalent), determination of est rs, determination of nitro, nitroso, and nitrate groups, applications of lithium aluminum hydride to organic analysis, methods based on reaction rate, phase solubility analysis, and countercurrent distribution.

Each chapter has been written by a specialist in the field covered. It is interesting to note that the chapter on the applications of lithium aluminum hydride to organic analysis was contributed by Dr. Takeru Higuchi of the School of Pharmacy at the University of Wisconsin.

Volume two of *Organic Analysis* includes references to the original literature and is provided with an unusually complete subject index.

Compounds with Condensed Thiophene Rings. By H. D. HARTOUGH and S. L. MEISEL. Seventh volume in the series *The Chemistry of Heterocyclic Compounds.* Interscience Publishers Inc., New York, 1954. 515 pp. 16.5 x 24 cm. Price $16.50; Subscription Price $15.

This seventh volume of a series of comprehensive monographs on the chemistry of heterocyclic compounds is intended to supplement the third book in the series, *Thiophene and Its Derivatives*, which was described [THIS JOURNAL, **41**, 338(1952)] previously. The chemistry of compounds, containing the condensed thiophene nucleus, was originally scheduled to be included in the aforementioned volume. The large number of condensed substances containing the thiophene nucleus was so great and diverse that two volumes were found to be preferable for a satisfactory treatment of the subject.

This supplementary volume is divided into nine chapters, covering the following subjects: factors influencing chemical reactivity and substitution of thiophene systems, thionaphthene and other thiophene compounds containing one carbocyclic fused ring, thioindigo and related dyes, dibenzothiophene and its derivatives, naphthothiophenes and other thiophene compounds containing two carbocyclic fused rings, thiophene systems containing three or more carbocyclic fused rings, condensed thiophenes containing O, S, and Hg ring systems, condensed thiophene compounds containing nitrogen ring systems, and condensed selenophene and tellurophene systems.

The book is documented with all references to the original literature available up to May 15, 1952. Its value is enhanced by a patent bibliography, a ring index, and an entirely adequate subject index. The printing and binding are of good quality.

Organic Syntheses. Vol. 34. WILLIAM S. JOHNSON, Editor-in-Chief. John Wiley & Sons, Inc., New York, 1954. vi + 121 pp. 15.5 x 23.5 cm. Price $3.50.

This anpual publication of tested methods for the preparation of organic compounds, now in its thirty-fourth year, follows the design of previous editions. This series is so well known, and so highly respected by organic chemists and research workers in other fields, who from time to time are required to synthesize certain compounds, for which procedures are given in *Organic Syntheses*, that it requires no particular treatment other than to list the compounds covered. These are as follows: 2-*p*-acetylphenylhydroquinone; azelanitrile; β-(*o*-carboxyphenyl)-propionic acid; cetylmalonic ester; 2-chloro-1,1,2-trifluorethyl ethyl ether; cycloheptanone; di-*tert*-butyl malonate; 3,4-dihydro-2-methoxy-4-methyl-2H-pyran; 9,10-dihydrophenanthrene; *p,p'*-dinitrobibenzyl, 1,4-dinitrobutane; dimethylfurazan; diphenylacetylene; diphenyl succinate; ethoxyacetylene; ethyl chlorofluoroacetate; ethyl enanthylsuccinate; ethyl β,β-pentamethyleneglycidate, hemimellitene; *o*-methylbenzyl alcohol; 2-methybenzyldimethylamine; N-methyl-1,2-diphenylethylamine and hydrochloride; methylisourea hydrochloride; 3 - methyl - 1,5 - pentanediol; 3 - methylthiophene; phenanthrenequinone; 1-phenylpiperidine; *o*-phthalaldehyde; sodium β-styrenesulfonate and β-styrenesulfonyl chloride; tetralin hydroperoxide; *p*-toluenesulfinyl chloride, *p*-tolylsulfonylmethylnitrosamide; and *o*-xylylene dibromide.

BOOKS RECEIVED

Principles of Internal Medicine. T. R. HARRISON *et al.*, Editors. 2nd ed. The Blakiston Co., Inc., New York, 1954. 23 + 1703 + 87 pp. 26.5 x 20 cm. Price, Student 1 Vol. ed. $16., Professional 2 Vol. ed., boxed, $21.

Hugh Roy Cullen. A Story of American Opportunity. By ED KILMAN and THEON WRIGHT. Illustrated by Nick Eggenhoffer. Prentice-Hall, Inc., New York, 1954. viii + 376 pp. 15.5 x 23 cm. Price $4.

Pathogenesis of Cancer. By JOHN E. GREGORY, Fremont Foundation Publishers, Pasadena, 2nd ed., 1952. 202 pp. 23.5 x 16 cm. Price $7.50.

Pharmacology in Medicine. A Collaborative Textbook. Edited by VICTOR A. DRILL. McGraw-Hill Book Company, Inc., New York, 1954. xii + 87 sections + 26 pp. 29 x 23 cm. Price $19.50.

Organic Analysis. Vol. 2. Editors: JOHN MITCHELL, JR., I. M. KOLTHOFF, E. S. PROSKAUER, A. WEISSBERGER. Interscience Publishers, Inc., New York, 1954. vii + 372 pp. 16 x 23.5 cm. Price $8.50.

Organic Syntheses. Vol. 34. WILLIAM S. JOHNSON, Editor-in-Chief. John Wiley & Sons, Inc., New York, 1954. vi + 121 pp. 15.5 x 23.5 cm. Price $3.50.

The Vitamins—Chemistry, Physiology, Pathology. Vol. III. W. H. SEBRELL, JR., and ROBERT S. HARRIS, Editors. Academic Press, Inc., New York, 1954. xv + 665 pp. 16 x 23.5 cm. Price $15.

Good formulation starts with the best active ingredients. When you start with Reheis, you are working with a manufacturer whose quality is recognized as the accepted standard for alumina gels and basic aluminum salts. In addition, the Reheis wide variety of forms permits you to develop more saleable, more individualized products. Why not plan to formulate with Reheis fine chemicals as the active ingredients for your preparations.

1345

REHEIS COMPANY, INC.
Manufacturers of Fine Chemicals
BERKELEY HEIGHTS · NEW JERSEY

· Consultation · Research
ew Drugs
Toxicological Studies
elopment · Special Analyses
ion · Phenol Coefficients
LaWall & Harrisson
Div. P, 1921 Walnut St., Philadelphia 3, Pa.
Bacteriologists · Chemists · Pharmacologists

DRUG STANDARDS

A Publication of
The American Pharmaceutical Association

Single copies $1.00
Annual Subscription $5.00

Order from

The American Pharmaceutical Association
2215 Constitution Avenue
Washington 7, D. C.

Handsome Durable Binders for Journals of the American Pharmaceutical Association

Combination Set for Practical Pharmacy and Scientific Editions $5.50 a pair
For Single Editions $3.25 each

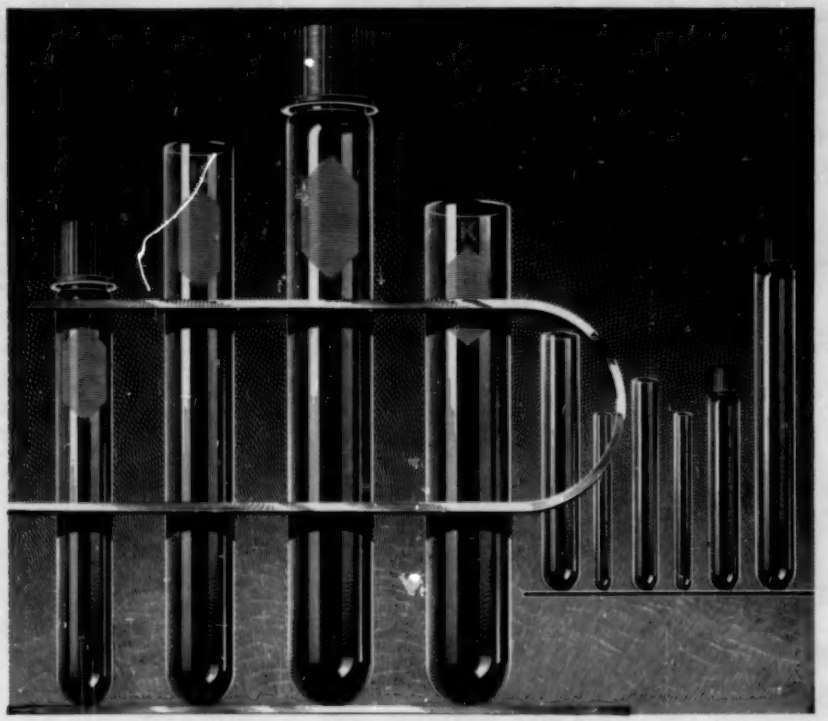
These binders open flat as a bound book. Made of durable imitation leather, they will preserve your journals permanently. Each cover holds 12 issues (one volume)....Do your own binding. Instructions easy to follow. Mail coupon for binders on 10 day trial.

.....Mail Coupon Today.....

SUCKERT LOOSE-LEAF COVER CO.
234 W. LARNED ST. DETROIT 26, MICH.

Send....binders for the Journal of the American Pharmaceutical Association.
....Practical Pharmacy Edition for years....Vols.....
....Scientific Edition for years.....Vols.....
....Combination for years.....Vols.....
Will remit $..... in 10 days or return binders.

Name .....
Address .....
City.....Zone.....State.....
Postage prepaid in U. S.—Foreign Extra



Kimble Culture Tubes are available with three types of caps: (1) cemented in pulp-backed vinylite liner; (2) cemented in rubber liner; (3) pressure fitted Teflon liner.

DURABLE!

KIMBLE CULTURE TUBES

Their long, useful life means low costs for you

Kimble Culture Tubes are made of Kimble N51A glass—a borosilicate glass with outstanding resistance to chemical attack. They do not affect the growth or viability of cultures. They resist the chemical attack of washing solutions. Kimble Culture Tubes resist alkaline, acid and nearly neutral solutions, including hot water and steam.

Tubes easily withstand heat-shock of sterilization . . . are thoroughly annealed. Walls are of uniform thickness . . . bottoms are of ample weight to avoid "breaking-out."

New style, longer caps, when slightly loosened, permit free exchange between the atmospheres inside and outside the tubes, completely satisfying the gas requirements of aerobic cultures. Threads are so designed that there is no danger of the slightly loosened caps falling off.

See your laboratory supply dealer for information about Culture Tubes and the complete Kimble glassware line. Or write Kimble Glass Company, subsidiary of Owens-Illinois, Box 1035, Toledo 1, Ohio.

KIMBLE LABORATORY GLASSWARE
AN (O) PRODUCT

OWENS-ILLINOIS
GENERAL OFFICES · TOLEDO 1, OHIO